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MANUAL OF BACTERIOLOGY

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MANUAL OF BACTERIOLOGY

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PREFACE TO THE EIGHTH EDITION

By the death of my friend, Professor James Ritchie, I have been deprived of the services of him who worked so long and so devotedly with me in the preparation of the book, and, in considering the question of a new edition, I felt that it was scarcely possible for me to carry it out satisfactorily by myself. To meet the requirements of the situation thus arising, I have been fortunate in obtaining the co-operation of Professor Browning and Professor Mackie for the revision, and I desire to express my high appreciation of the character of their work and of the whole-hearted manner in which they have carried it out.

The period since the last edition appeared has been characterised not so much by outstanding discoveries as by steady growth in every branch of the subject, associated with the development of improved technique. Comparatively large additions and alterations will be found in nearly every chapter. Some portions have been rewritten and a certain rearrangement of the subjects has been carried out ; other portions now of less importance have been condensed, and others again have been put in smaller type. Thus the actual size of the book has not been greatly increased.

A number of new illustrations have been added, whilst a few have been discarded. As we have indicated in the text, we owe some of these new illustrations to the kindness of Dr. Byam, Dr. Archibald, Mr. Clifford Dobell, Professor Warrington Yorke,

and Dr. George Buchanan, and to them we have pleasure in recording our thanks. We desire to thank also Professor Percy Groom for revising his introduction to the Pathogenic Fungi. We are indebted to Messrs. E. and S. Livingstone for permission to use an illustration (Fig. 21) from one of their publications. The other new microphotographs and drawings are by Mr. John Kirkpatrick of the Pathological Department, The University, Glasgow.

R. M.

February 1927.

PREFACE TO THE FIRST EDITION

THE science of Bacteriology has, within recent years, become so extensive, that in treating the subject in a book of this size we are necessarily restricted to some special departments, unless the description is to be of a superficial character. Accordingly, as this work is intended primarily for students and practitioners of medicine, only those bacteria which are associated with disease in the human subject have been considered. We have made it a chief endeavour to render the work of practical utility for beginners, and, in the account of the more important methods, have given elementary details which our experience in the practical teaching of the subject has shown to be necessary.

In the systematic description of the various bacteria, an attempt has been made to bring into prominence the evidence of their having an etiological relationship to the corresponding diseases, to point out the general laws governing their action as producers of disease, and to consider the effects in particular instances of various modifying circumstances. Much research on certain subjects is so recent that conclusions on many points must necessarily be of a tentative character. We have, therefore, in our statement of results aimed at drawing a distinction between what is proved and what is only probable.

In an Appendix we have treated of four diseases ; in two of these the causal organism is not a bacterium, whilst in the other two its nature is not yet determined. These diseases have been

included on account of their own importance and that of the pathological processes which they illustrate.

Our best thanks are due to Professor Greenfield for his kind advice in connection with certain parts of the work. We have also great pleasure in acknowledging our indebtedness to Dr. Patrick Manson, who kindly lent us the negatives or preparations from which Figs. 174–179 have been executed.

As we are convinced that to any one engaged in practical study, photographs and photomicrographs supply the most useful and exact information, we have used these almost exclusively in illustration of the systematic description. These have been executed in the Pathological Laboratory of the University of Edinburgh by Mr. Richard Muir. The line drawings were prepared for us by Mr. Alfred Robinson, of the University Museum, Oxford.

To the volume is appended a short Bibliography, which, while having no pretension to completeness, will, we hope, be of use in putting those who desire further information on the track of the principal papers which have been published on each of the subjects considered.

June 1897.

CONTENTS

CHAPTER I

General Morphology and Biology

PAGE

Introductory—Structure of the bacterial cell—Reproduction of bacteria—Spore formation—Motility—Minuter structure of the bacterial protoplasm—Chemical composition of bacteria—Classification—Food supply—Relation of bacteria to moisture, gaseous environment, temperature, and light—Conditions affecting bacterial motility—Effects of bacteria in nature—Methods of bacterial action—Variation and mutation—Bacteriophage—Nature of bacteriophage 1

CHAPTER II

Methods of Cultivation of Bacteria

Introductory—Methods of sterilisation—Preparation of culture media—Use of the culture media—Methods of the separation of aerobic organisms—Principles of the culture of anaerobic organisms—Miscellaneous methods—General laboratory rules 33

CHAPTER III

Microscopic Methods

The microscope—Examination of hanging-drop cultures—Film preparations—Examination of bacteria in sections—Staining principles—Mordants and decolorisers—Formulæ of stains—Gram's method and its modifications—Stain for tubercle and other acid-fast bacilli—Staining of spores, capsules, and flagella—The Romanowsky stains—Fixation and hardening of tissues 95

CHAPTER IV

EXAMINATION OF SERUM—PREPARATION OF VACCINES—INOCULATION OF ANIMALS—METHODS OF OBTAINING BACTERIOLOGICAL MATERIAL FOR EXAMINATION

PAGE

Observation of agglutination and sedimentation—Opsonic methods—Method of measuring the phagocytic capacity of the leucocytes—Bactericidal methods—Hæmolytic tests—Fixation and deviation of complement—Wassermann reaction—Flocculation reaction—Preparation of vaccines—Methods of counting bacteria in dead cultures—Inoculation of animals—Autopsies on animals—Methods of obtaining pathological material 122

CHAPTER V

RELATIONS OF BACTERIA TO DISEASE—THE PRODUCTION OF TOXINS BY BACTERIA

Introductory—Conditions modifying pathogenicity—Carriers—Modes of bacterial action—Tissue changes produced by bacteria—Local lesions—General lesions—Disturbances of metabolism by bacterial action—The production of toxins by bacteria, and the nature of these—Allied vegetable and animal poisons—The theory of toxic action . 150

CHAPTER VI

IMMUNITY

Introductory—Acquired immunity—Artificial immunity—Active and local immunity—Antibodies and their specificity—Antitoxic serum—Nature of antitoxic action—Antibacterial serum—Hæmolytic and other sera—Opsonic action—Agglutination—Precipitins—Nature of agglutination and precipitation—Source and nature of antibodies—Non-specific formation of antibodies—Theories as to acquired immunity—Ehrlich's side-chain theory—Theory of phagocytosis—Natural immunity—Anaphylaxis—Allergy—Serum disease in man 175

CHAPTER VII

INFLAMMATORY AND SUPPURATIVE CONDITIONS

The relations of inflammation and suppuration—The bacteria of inflammation and suppuration—Experimental inoculation—Lesions in the human subject—Mode of entrance

PAGE

and spread of pyogenic bacteria—Ulcerative endocarditis —Acute suppurative periostitis—Erysipelas—Puerperal sepsis — Acute rheumatism — Conjunctivitis — Scarlatina — Dick reaction — Vaccination treatment of infections by the pyogenic cocci—Methods of examination in inflammatory and suppurative conditions . . . 233

CHAPTER VIII

INFLAMMATORY AND SUPPURATIVE CONDITIONS (*continued*): THE ACUTE PNEUMONIAS, EPIDEMIC CEREBRO-SPINAL MENINGITIS

PNEUMONIA: Introductory—Historical—Cultivation of pneumococcus—Experimental inoculation—Lesions caused by pneumococcus—Immunity phenomena—Strains of pneumococcus—Methods of examination—Friedländer's pneumobacillus. EPIDEMIC CEREBRO-SPINAL MENINGITIS: Characters of meningococcus—Serum reactions—Allied diplococci 268

CHAPTER IX

GONORRHŒA AND SOFT SORE

GONORRHŒA: Microscopical characters of gonococcus—Cultivation—Comparison with meningococcus—Relations to the disease—Its toxin—Distribution—Gonococcus in joint affections—Methods of diagnosis. SOFT SORE: Microscopical characters and cultivation of bacillus . . 299

CHAPTER X

TUBERCULOSIS

Historical—Tuberculosis in animals—Tubercle bacillus—Staining reactions—Cultivation of tubercle bacillus—Powers of resistance—Action on the tissues—Histology of tuberculous nodules—Distribution of bacilli—Bacilli in tuberculous discharges—Experimental inoculation—Varieties of tuberculosis—Other acid-fast bacilli—Action of dead tubercle bacilli—Sources of human tuberculosis—Specific reactions of the tubercle bacillus—Phenomena of supersensitiveness—Tuberculin reactions—Immunity phenomena in tuberculosis—Therapeutic application of the tuberculins—Varieties of tuberculin—Antitubercular sera—Methods of examination 311

CHAPTER XI

LEPROSY

PAGE

Pathological changes—Bacillus of leprosy—Position of the bacilli—Relations to the disease—Methods of diagnosis . 344

CHAPTER XII

GLANDERS, MELIOIDOSIS, AND RHINOSCLEROMA

GLANDERS: The natural disease—The glanders bacillus—Cultivation of glanders bacillus—Powers of resistance—Experimental inoculation—Action on the tissues—Mode of spread—Serum reactions—Mallein and its preparation—Methods of examination. MELIOIDOSIS. RHINOSCLEROMA 353

CHAPTER XIII

ACTINOMYCOSIS AND ALLIED DISEASES

Characters of the actinomyces—Tissue lesions—Distribution of lesions—Cultivation of actinomyces—Varieties of actinomyces and allied forms—Methods of examination and diagnosis—Leptothrix—Madura disease . . . 364

CHAPTER XIV

ANTHRAX

Historical summary—Bacillus anthracis—Appearances of cultures—Biology—Sporulation—Natural anthrax in animals—Experimental anthrax—Anthrax in man—Pathology—Mode of spread in nature—Immunisation of animals against anthrax—Methods of examination—Organisms biologically allied to anthrax bacillus . . 380

CHAPTER XV

TYPHOID FEVER—BACILLI ALLIED TO THE TYPHOID BACILLUS

Introductory—Bacillus coli—Culture reactions—Isolation and recognition of B. coli—Varieties—Pathogenic properties—Bacillus typhosus—Appearances of cultures—Biological reactions—Pathological changes in typhoid fever

PAGE

—Immunisation of animals—Etiological relationships of bacillus typhosus—Typhoid carriers—Epidemiology of typhoid fever—Serum diagnosis of typhoid fever—Vaccination against typhoid—Paratyphoid fever—The paratyphoid bacillus—Bacillus enteritidis of Gaertner—The psittacosis bacillus—Danysz's bacillus and rat viruses—Bacillary dysentery—Infantile diarrhœa—Differentiation of coli-typhoid group by culture and agglutination—Characters of bacilli of coli-typhoid group . . 397

CHAPTER XVI

THE VIBRIO CHOLERÆ AND ALLIED ORGANISMS

Introductory—Characters of cholera vibrio—Distribution within body—Cultivation—Reactions—Experimental inoculation—Immunity—Allied organisms—Anti-cholera inoculation—Methods of diagnosis . . . 445

CHAPTER XVII

DIPHTHERIA

Historical—General facts—Bacillus diphtheriæ—Microscopical characters—Cultivation—Powers of resistance—Distribution—Inoculation experiments—Toxins of diphtheria bacillus—Variations in virulence and in toxin production—Carriers—Immunity—Bacilli allied to the diphtheria bacillus—Summary of pathogenic action—Methods of diagnosis 462

CHAPTER XVIII

EPIDEMIC INFLUENZA, INFECTIOUS CORYZA, WHOOPING-COUGH (PERTUSSIS)

EPIDEMIC INFLUENZA: Characters, cultivation, and distribution of influenza bacillus—Methods of examination—Question of a filterable virus—Bacterium pneumosintes. INFECTIOUS CORYZA. WHOOPING-COUGH: Characters of bacillus—Pathogenic effects—Methods of examination . 486

CHAPTER XIX

PLAGUE AND TULARÆMIA

PLAGUE: The plague bacillus—Cultivation—Anatomical changes and distribution of bacilli—Experimental inoculation—Paths and modes of infection—Toxins, im-

PAGE

munity, etc.—Preventive inoculation—Anti-plague sera —Methods of diagnosis—Recognition of infection in rats —Pasteurella group. TULARÆMIA 501

CHAPTER XX

MALTA FEVER AND EPIZOOTIC ABORTION

MALTA FEVER: Bacillus melitensis—Relations to the disease —Mode of spread—Methods of diagnosis. EPIZOOTIC ABORTION 517

CHAPTER XXI

TETANUS—OTHER ANAEROBIC BACILLI

TETANUS: Introductory—Historical—Bacillus tetani—Occurrence in nature—Isolation—Characters of cultures and conditions of growth—Pathogenic effects—Experimental inoculation—Tetanus toxins—Anti-tetanic serum—Methods of examination—BACILLUS BOTULINUS. ANAEROBES IN INFECTED WOUNDS. BACILLUS WELCHII: Microscopical characters—Cultivation—Pathogenic effects —Experimental inoculation. MALIGNANT ŒDEMA: Microscopical characters of bacillus—Cultivation—Experimental inoculation—Immunity. QUARTER-EVIL. FUSIFORM ANAEROBIC BACILLI. 524

CHAPTER XXII

DISEASES DUE TO SPIROCHÆTES: SYPHILIS AND YAWS

SYPHILIS: Microscopic characters of Treponema pallidum—Distribution—Cultivation—Transmission of the disease—Serum diagnosis—Wassermann and flocculation reactions. FRAMBŒSIA or YAWS 562

CHAPTER XXIII

DISEASES DUE TO SPIROCHÆTES (*continued*): RELAPSING FEVER AND AFRICAN TICK FEVER, ETC.

RELAPSING FEVER: Characters of the Spirochæte—Relations to the disease—Immunity—Varieties. AFRICAN RELAPSING FEVER. SPIROCHÆTAL or INFECTIVE JAUNDICE:

PAGE

Morphology of the spirochæte—Cultivation—Relation to the disease—Experimental inoculation. YELLOW FEVER: Etiology—Epidemiology. RAT-BITE FEVER. PHLEBOTOMUS FEVER. SEVEN-DAY FEVER. DENGUE . . . 576

CHAPTER XXIV

RICKETTSIA INFECTIONS

Introductory—TYPHUS FEVER—ROCKY MOUNTAIN FEVER—TRENCH FEVER 601

CHAPTER XXV

FILTER-PASSING VIRUSES: SMALLPOX, HYDROPHOBIA, MEASLES

Introductory—SMALLPOX: Jennerian vaccination—Relation of smallpox to cowpox—Nature of virus—Antibodies. HYDROPHOBIA or RABIES: Introductory—Pathology—The virus of hydrophobia—Prophylaxis—Antirabic serum—Methods. MEASLES 610

CHAPTER XXVI

FILTER-PASSING VIRUSES (*continued*): EPIDEMIC POLIOMYELITIS, EPIDEMIC ENCEPHALITIS, HERPES

EPIDEMIC POLIOMYELITIS: Infectivity—Nature of virus—Pathology—Immunity phenomena. EPIDEMIC ENCEPHALITIS. HERPES: Etiology—Relations and biological nature of virus 634

CHAPTER XXVII

PROTOZOAL DISEASES: MALARIA, AMŒBIC DYSENTERY

MALARIA: Characters of parasites—Sexual cycle in the mosquito—Varieties of parasites—Pathology—Methods of examination. AMŒBIC DYSENTERY: Etiology—Distribution of Entamœba histolytica—Experimental inoculation—Methods of examination 647

CHAPTER XXVIII

PROTOZOAL DISEASES (*continued*) : TRYPANOSOMIASIS, LEISHMANIASIS, ETC.

PAGE

THE PATHOGENIC TRYPANOSOMES : Morphology and biology—Sleeping sickness—Trypanosoma gambiense—Trypanosoma rhodesiense—Methods of examination—Trypanosoma cruzi—Nagana or tsetse fly disease—Trypanosoma lewisi. LEISHMANIASIS : Leishmania donovani—Leishmania infantum—Leishmania tropica. PIROPLASMOSIS. OROYA FEVER 676

CHAPTER XXIX

PATHOGENIC FUNGI

Botanical description—Methods—Microspora—Trichophyta—Achoria—Thrush—Aspergillosis—Sporotrichosis—Hemisporosis—Blastomycosis—Microsporon furfur . 707

APPENDIX

THE BACTERIOLOGY OF AIR, SOIL, WATER, SEWAGE, AND MILK. ANTISEPTICS

AIR : Methods of examination. SOIL : Methods of examination—Varieties of bacteria in soil. WATER : Methods of examination—Bacteria in water—Isolation of typhoid-paratyphoid group and vibrio of cholera. SEWAGE : Purification. MILK : Souring of milk—Pathogenic organisms in milk—Sterilisation of milk. ANTISEPTICS : Methods of investigation—Action of antiseptics—Effects of certain antiseptics 731

BIBLIOGRAPHY 761

INDEX 805

LIST OF COLOURED PLATES

PLATE I.

FIG.

1. Film of pus, containing staphylococci and streptococci.
2. Fraenkel's pneumococcus in sputum.
3. Meningococcus in epidemic cerebro-spinal fever.
4. Film from a scraping of throat in Vincent's angina, showing fusiform bacilli and spirochætes.
5. Gonorrhœal pus, showing gonococci and staphylococci.

PLATE II.

6. Treponema pallidum, case of congenital syphilis.
7. Tubercle bacilli and other bacteria in sputum.
8. Leprous skin, showing clumps of bacilli in the cutis.
9. Leprous granulation tissue, showing bacilli.

PLATE III.

10. Streptothrix actinomyces.
11. Anthrax bacilli.
12. Bacillus diphtheriæ.
13. Bacillus diphtheriæ (involution forms).
14. Hofmann's pseudo-diphtheria bacillus.
15. Typhoid bacilli, showing flagella.

PLATE IV.

FIG.

16. Negri bodies in nerve cells in rabies.
17. Bacillus pestis (involution forms).
18. Spirochæte of relapsing fever.
19. Vibrio choleræ, showing flagella.
20. Bacillus tetani, showing spores.

PLATE V.

21. The parasite of benign tertian malaria.
    Cycle I. (Schizogony). Asexual cycle in human blood.
    Cycle II. (Sporogony). Sexual cycle in the mosquito.
22. The parasite of malignant malaria.

PLATE VI.

23. Entamœba histolytica in pus, from tropical abscess of liver
24. Leishman-Donovan bodies, from a case of kala-azar.
25. Trypanosoma gambiense.

LIST OF ILLUSTRATIONS IN TEXT

FIG.		PAGE
1.	Forms of bacteria	15
2.	Growth of staphylococcus albus on agar, before and after inoculation with bacteriophage	29
3.	Plate culture of staphylococcus with "colonies" of bacteriophage	30
4.	Hot-air steriliser	34
5.	Koch's steam steriliser	35
6.	Autoclave	37
7.	Comparator used in estimation of hydrogen-ion concentration	46
8.	Hot-water funnel	49
9.	Blood serum inspissator	52
10.	Cylinder of potato cut obliquely	57
11.	Ehrlich's tube, containing piece of potato	57
12.	Tubes for demonstrating gas-formation by bacteria	65
13.	Apparatus for filling tubes	68
14.	Tubes of media	68
15.	Platinum wires in glass handles	69
16.	Method of making a stab culture	70
17.	Rack for platinum needles	70
18.	Hollow-ground slides for hanging-drop cultures	71
19.	Petri's capsule	73
20.	Diagram of method of inoculating plate from fæces	75
21.	Arrangement of bottles for reducing pressure in hydrogen supply from cylinder	78
22.	Apparatus for supplying hydrogen for anaerobic cultures	79
23.	Tubes for anaerobic cultures on the surface of solid media	79
24.	M'Leod's capsule for anaerobic plating	80
25.	Henry's apparatus	81
26.	Bulloch's apparatus for anaerobic plate cultures	81
27.	Top of M'Intosh and Fildes' anaerobic jar	82
28.	Flask for anaerobes in liquid media	84
29.	Flask arranged for culture of anaerobes which develop gas	84
30.	Hearson's incubator for use at 37° C.	86
31.	Geissler's vacuum pump for filtering cultures	87
32.	Chamberland's candle and flask arranged for filtration	88
33.	Chamberland's bougie with lamp funnel	88
34.	Another form of arrangement of filtering candle and flask	89
35.	Muencke's modification of Chamberland's filter	89
36.	Flask for filtering small quantities of fluid	90
37.	Geryk air-pump for drying *in vacuo*	91
38.	Diagram showing course of rays in dark-ground illumination	97

FIG		PAGE
39.	Cornet's forceps for holding cover-glasses	99
40.	Siphon wash-bottle for distilled water	103
41.	Wright's blood-capsule	147
42.	Test-tube and pipette arranged for obtaining fluids containing bacteria	149
43.	Staphylococcus pyogenes aureus, young culture on agar. ×1000	236
44.	Two stab cultures of staphylococcus pyogenes aureus in gelatin	236
45	Streptococcus pyogenes; young culture on agar. ×1000	238
46.	Culture of the streptococcus pyogenes on an agar plate	238
47.	Micrococcus tetragenus; young culture on agar. ×1000	244
48	Bacillus pyocyaneus; young culture on agar. ×1000	246
49	Streptococci in acute suppuration. ×1000	249
50	Film from urinary sediment, showing B. coli ×1000	251
51.	Minute focus of commencing suppuration in brain ×50	252
52.	Secondary infection of a glomerulus of kidney by the staphylococcus aureus ×300	253
53.	Section of a vegetation in ulcerative endocarditis. ×600	255
54.	Film preparation from a case of acute conjunctivitis, showing Koch-Weeks bacilli. ×1000	260
55.	Koch-Weeks bacillus, from a young culture on blood agar. ×1000	261
56.	Film preparation of conjunctival secretion, showing the Morax diplo-bacillus of conjunctivitis. ×1000	261
57.	Film preparation of pneumonic sputum, showing numerous pneumococci (Fraenkel's). ×1000	269
58.	Fraenkel's pneumococcus in serous exudation. ×1000	270
59	Stroke culture of Fraenkel's pneumococcus on blood agar	271
60.	Fraenkel's pneumococcus, from a pure culture on blood agar. ×1000	271
61.	Capsulated pneumococci in blood taken from the heart of a rabbit. ×1000	275
62.	Friedländer's pneumobacillus, from exudate in a case of pneumonia. ×1000	286
63	Stab culture of Friedländer's pneumobacillus	287
64.	Friedländer's pneumobacillus, from a culture on agar. ×1000	287
65.	Film preparation of exudation from a case of meningitis. ×1000	288
66.	Two-day colonies of the meningococcus on Martin's medium. ×9	289
67.	Pure culture of meningococcus	290
68.	Portion of film of gonorrhœal pus. ×1000	300
69.	Colonies of gonococcus on serum agar. ×9	301
70.	Gonococci, from a pure culture on blood agar. ×1000	301
71.	Film preparation of pus from soft chancre, showing Ducrey's bacillus. ×1500	308

FIG		PAGE
72.	Ducrey's bacillus. ×1500	309
73.	Tubercle bacilli, from a pure culture on glycerin agar. ×1000	313
74.	Tubercle bacilli in phthisical sputum. ×1000	314
75.	Cultures of tubercle bacilli on glycerin agar	317
76.	Tubercle bacilli in section of human lung in acute phthisis. ×1000	320
77.	Tubercle bacilli in giant-cells. ×1000	321
78.	Tubercle bacilli in urine ×1000	322
79.	Bovine tubercle bacilli in milk. ×1000	324
80	Cultures of bovine and human tubercle bacilli, 5 weeks old, on glycerin egg medium	325
81.	Moeller's Timothy-grass bacillus. ×1000	329
82.	Cultures of acid-fast bacilli grown at room temperature	330
83.	Smegma bacilli. ×1000	331
84	Section through leprous skin, showing the masses of cellular granulation tissue in the cutis. ×80	345
85.	Superficial part of leprous skin. ×500	347
86.	High-power view of portion of leprous nodule, showing the arrangement of the bacilli within the cells of the granulation tissue ×1100	348
87.	Glanders bacilli. ×1000	355
88	Glanders bacilli, from a pure culture on glycerin agar. ×1000	356
89	Low-power view of untreated colony of actinomyces in pus	365
90.	Actinomycosis of human liver. ×500	366
91	Actinomyces in human kidney. ×500	367
92.	Colonies of actinomyces. ×60	368
93.	Cultures of streptothrix actinomyces on glycerin agar	371
94.	Actinomyces, from a culture on glycerin agar. ×1000	372
95.	Shake cultures of actinomyces in glucose agar	373
96.	Section of a colony of actinomyces, from a culture in blood serum. ×1500	373
97.	Streptothrix maduræ. ×1000	378
98	Surface colony of the anthrax bacillus on an agar plate. ×30	382
99.	Anthrax bacilli, arranged in chains, from a twenty-four hours' culture on agar at 37° C. ×1000	382
100.	Stab culture of the anthrax bacillus in peptone-gelatin	383
101.	Anthrax bacilli containing spores. ×1000	384
102.	Scraping from spleen of guinea-pig dead of anthrax. ×1000	387
103.	Portion of kidney of a guinea-pig dead of anthrax. ×300	388
104.	Bacillus coli. ×1000	398
105.	A large clump of typhoid bacilli in spleen. ×500	406

FIG.		PAGE
106.	Typhoid bacilli, from a young culture on agar, showing some filamentous forms. ×1000	407
107.	Typhoid bacilli, from a young culture on agar, showing flagella. ×1000	407
108.	Culture of the typhoid bacillus and of the bacillus coli	408
109.	Colonies of the typhoid bacillus on a gelatin plate. ×15	409
110.	Smooth and rough types of colony of B. paratyphosus B. ×40	424
111.	Cholera vibrios, from a culture on agar of twenty-four hours' growth. ×1000	446
112.	Cholera vibrios stained to show the terminal flagella ×1000	446
113.	Cholera vibrios from an old agar culture. ×1000	447
114.	Puncture culture of the cholera vibrio	448
115.	Colonies of the cholera vibrio on a gelatin plate	449
116.	Vibrio metchnikovi. ×1000	459
117.	Puncture cultures in peptone gelatin	460
118.	Finkler and Prior's vibrio. ×1000	461
119.	Diphtheria bacilli, from a twenty-four hours' culture on agar. ×1000	463
120.	Diphtheria bacilli, from a three days' agar culture. ×1000	464
121.	Involution forms of the diphtheria bacillus. ×1000	464
122.	Cultures of the diphtheria bacillus on an agar plate	466
123.	Diphtheria colonies, two days old, on agar. ×8	467
124.	Film preparation from diphtheria membrane, showing numerous diphtheria bacilli. ×1000	468
125.	Section through a diphtheritic membrane in trachea, showing diphtheria bacilli. ×1000	469
126.	Pseudo-diphtheria bacillus (Hofmann's) ×1000	483
127.	Xerosis bacillus from a young agar culture. ×1000	483
128.	Film preparation of influenza sputum, showing influenza bacilli along with leucocytes. ×1000	487
129.	Colonies of influenza bacilli on blood-agar plate ×5	488
130.	Film preparation from young culture of influenza bacillus ×1000	488
131.	Film preparation from older culture of influenza bacillus, showing involution forms. ×1000	488
132	Film preparation from a twenty-four hours' culture of the whooping-cough bacillus. ×1000	497
133.	Film preparation from a plague bubo. ×1000	501
134.	Bacillus of plague from a young culture on agar. ×1000	502
135.	Bacillus of plague in chains. ×1000	502
136.	Culture of the bacillus of plague on 4 per cent. salt agar. ×1000	503
137.	Section of a human lymphatic gland in plague. ×50	505
138	Film preparation of spleen of rat after inoculation with the bacillus of plague ×1000	507

FIG.		PAGE
139.	Film preparation from lung of rabbit dead of pasteurella infection. ×1000	515
140.	Bacillus melitensis. ×1000	518
141.	Tetanus bacilli, some of which possess spores. ×1000	525
142.	Film preparation of discharge from wound in a case of tetanus, showing several tetanus bacilli of "drum-stick" form. ×1000	526
143.	Tetanus bacilli, showing flagella. ×1000	527
144.	Spiral composed of numerous twisted flagella of the tetanus bacillus. ×1000	528
145.	Stab culture of the tetanus bacillus in glucose gelatin	529
146.	Colonies of the tetanus bacillus on agar, seven days old. ×50	530
147.	Film taken from margin of spreading gas gangrene, showing B welchii. ×1000	545
148.	Film from necrosed muscle in gas gangrene ×1000	546
149.	Film from a pure culture of B. welchii	546
150.	Bacillus welchii, showing capsules. ×1000	547
151.	Film preparation from the affected tissues in a case of malignant œdema. ×1000	552
152	Bacillus of malignant œdema, showing spores ×1000	552
153.	Stab cultures in agar—tetanus bacillus, bacillus of malignant œdema, and bacillus of quarter-evil	553
154.	B. sporogenes, pure culture, showing subterminal spores. ×1000	556
155.	Bacillus of quarter-evil, showing spores. ×1000	558
156.	Film preparation from a case of Vincent's angina. ×1000	560
157 and 158	Film preparations from juice of hard chancre, showing Treponema pallidum. ×1000	564
159.	Film preparation from juice of hard chancre, showing Treponema pallidum. ×2000	564
160.	Section of spleen from a case of congenital syphilis, showing Treponema pallidum. ×2000	565
161.	Treponema microdentium. ×1000	566
162.	Treponema gracile. ×1000	566
163.	Treponema refringens. ×1000	567
164.	Treponema of yaws. ×1000	574
165.	Spirochæte of relapsing fever in human blood. ×about 1000	577
166.	Treponema obermeieri in blood of infected mouse. ×1000	578
167.	Film of human blood containing spirochæte of tick fever. ×1000	582
168.	Spirochæte of human tick fever (Tr. duttoni) in blood of infected mouse. ×1000	583
169.	Specimens of Leptospira icterohæmorrhagiæ. ×1000	586
170.	L. icterohæmorrhagiæ, as seen in a culture by dark-ground illumination	586

FIG.		PAGE
171.	Sp. morsus muris, as seen in blood films from experimentally infected guinea-pig. A × 1000. B × 2000	596
172–177.	Various phases of the benign tertian parasite	651
178–183.	Exemplifying phases of the malignant parasite	653
184.	Entamœba histolytica. × 600	664
185.	Typical specimens of the intestinal amœbæ of man. × 1500	665
186.	Typical specimens of the cysts of the intestinal amœbæ of man. × 1500	667
187.	Section of wall of liver abscess, showing an amœba of spherical form with vacuolated protoplasm. × 1000	671
188.	Trypanosoma gambiense from blood of guinea-pig. × 1000	683
189.	Pathogenic trypanosomes. × 1500	687
190.	Trypanosoma brucei from blood of infected rat Note in two of the organisms commencing division of micronucleus and undulating membrane. × 1000	692
191.	Leishman-Donovan bodies from spleen smear. × 1000	696
192.	Leishman-Donovan bodies within endothelial cell in spleen. × 1000	697
193.	Leishmania donovani. × 1000	699
194.	Pathogenic fungi	709
195.	Hair infected with Microsporon audouini × 500	714
196.	Microsporon audouini on Sabouraud's maltose agar	715
197.	Trichophyta on Sabouraud's medium	716
198.	Hair infected with large-spored ringworm. × 500	717
199.	Favus hair showing air channels left by mycelium. × 300	718
200.	Photographs of Achoria	719
201.	Photograph of drawing of scraping from favus scutula. × 250	720
202.	Edge of living colony of Sporotrichon beurmanni on agar hanging-drop. × 200	724
203.	Film from agar culture of Sporotrichon beurmanni. × 1025	725
204.	Growth of blastomyces in kidney of rabbit. × 1000	727
205.	Double-contoured bodies in tissues from one of Rixford and Gilchrist's cases. × 500	728
206.	Microsporon furfur : scraping from skin. × 1000	729
207.	Petri's sand-filter	732
208.	Bacillus acidophilus from twenty-four hours' growth on agar. × 1000	749
209.	Surface colony of Bacillus acidophilus on agar plate. × 100	749
210.	Bacillus bifidus, as seen in film preparation from fæces of an infant. × 1000	750
211.	Bacillus bifidus, from a three days' culture on agar. × 1000	750

PLATE I.

FIG. 1. Film of pus, containing staphylococci and streptococci. Stained by Gram's method. × 1000 diameters.

FIG. 2. Fraenkel's pneumococcus in sputum, from a case of acute pneumonia. Rd. Muir's method of capsule staining. × 1000 diameters.

FIG. 3. Meningococcus in epidemic cerebro-spinal fever, from lumbar-puncture fluid, showing some involution forms. Leishman's stain. × 1000 diameters.

FIG. 4. Film from a scraping of throat in Vincent's angina, showing fusiform bacilli and spirochætes. Leishman's stain. × 1000 diameters.

FIG. 5. Gonorrhœal pus, showing gonococci (stained red) and staphylococci. Gram's method. × 1000 diameters.

PLATE I.

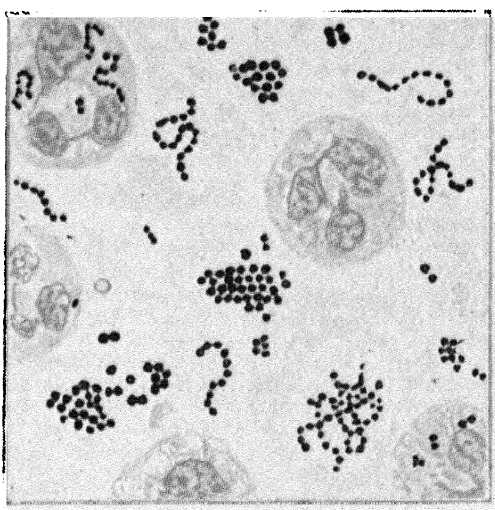


FIG. 1.

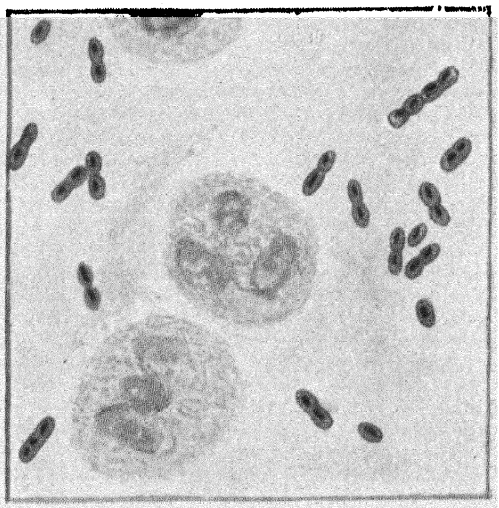


FIG. 2.

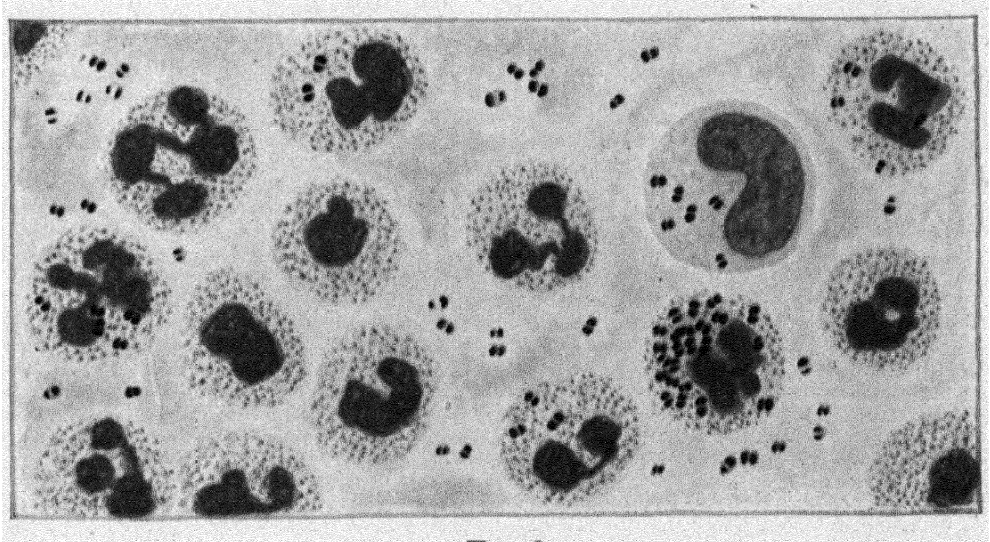


FIG. 3.

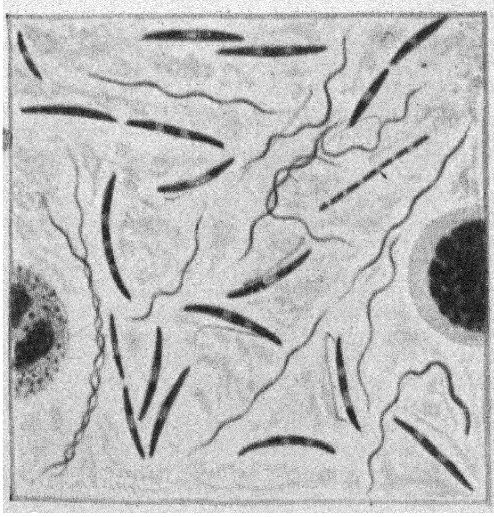


FIG. 4.

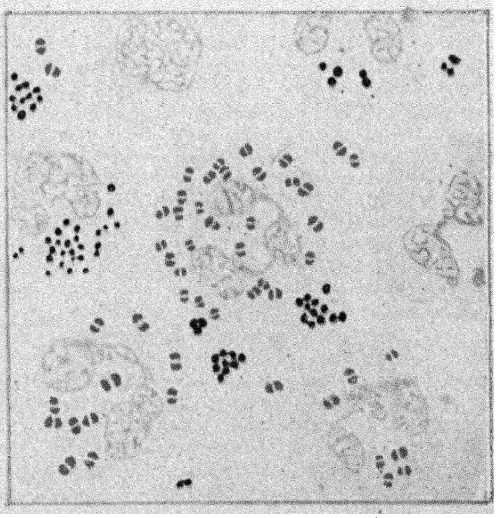


FIG. 5.

PLATE II.

FIG. 6. Treponema pallidum in section of spleen of child; case of congenital syphilis. Levaditi's stain. × 1000 diameters.

FIG. 7. Tubercle bacilli and other bacteria in sputum; case of pulmonary phthisis. Ziehl-Neelsen stain. × 1000 diameters.

FIG. 8. Section of leprous skin, showing numerous clumps of bacilli (stained red), in the cutis. Carbol-fuchsin and methylene-blue. × 80 diameters.

FIG. 9. Section of leprous granulation tissue, showing large numbers of bacilli, chiefly contained within cells. Carbol-fuchsin and methylene-blue. × 1000 diameters.

PLATE II.

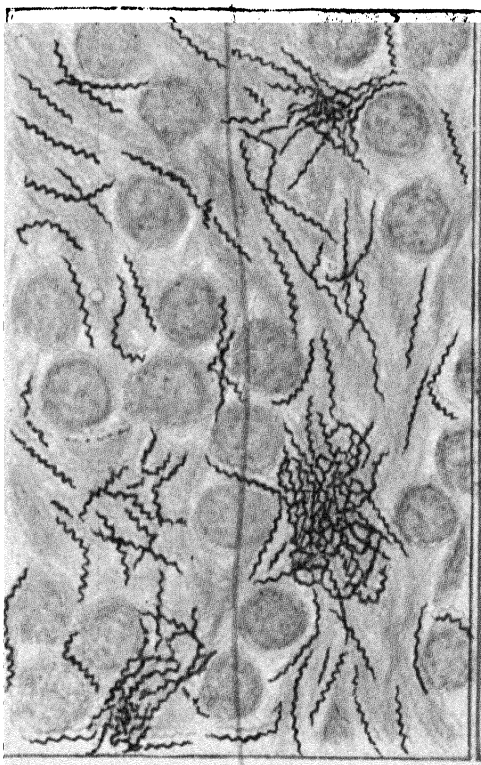


FIG. 6.



FIG. 7.

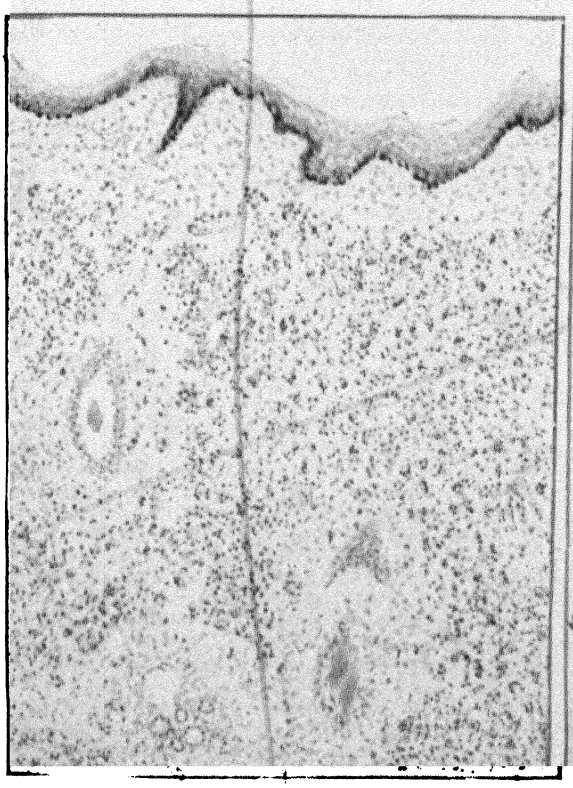


FIG. 8.



FIG. 9.

PLATE III.

FIG. 10. Streptothrix actinomyces, from agar culture. Gram's method. × 1000 diameters.

FIG. 11. Anthrax bacilli, from 4-days' agar culture showing spores. Carbol-fuchsin and methylene-blue. × 1000 diameters.

FIG. 12. Bacillus diphtheriæ, from a 12-hours' blood serum culture. Neisser's method with erythrosin counterstain. × 1000 diameters.

FIG. 13. Bacillus diphtheriæ, from a 5-days' blood serum culture, showing involution forms. Neisser's method with erythrosin counterstain. × 1000 diameters.

FIG. 14. Pseudo-diphtheria bacillus (Hofmann's), from young agar culture. Neisser's method with erythrosin counterstain.

To compare with Fig. 12. The morphological details are not well shown by this stain (*vide* Fig. 126, p. 483). × 1000 diameters.

FIG. 15. Typhoid bacilli from a 24-hours' agar culture, showing flagella. Rd. Muir's method. × 1000 diameters.

PLATE III.



FIG. 10.

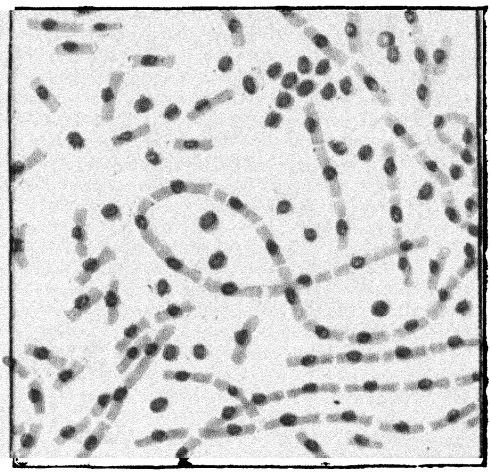


FIG. 11.

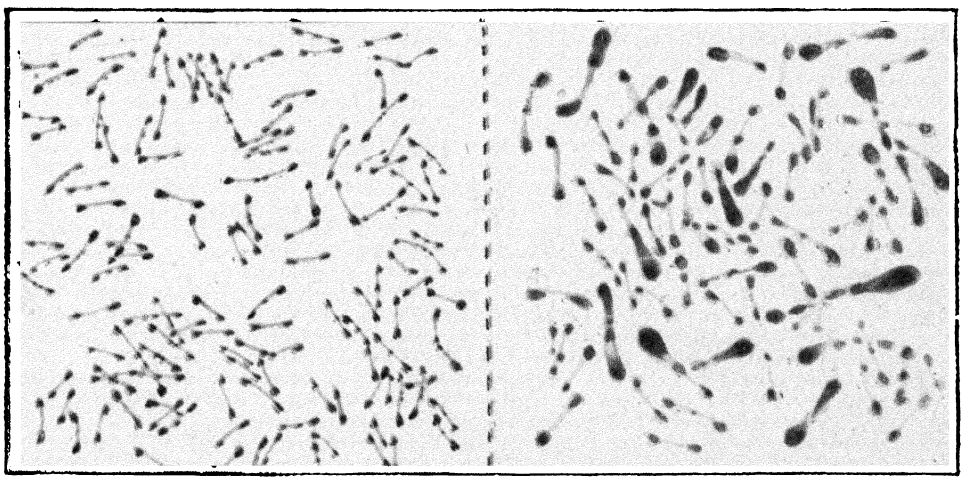


FIG. 12.

FIG. 13.



FIG. 14.

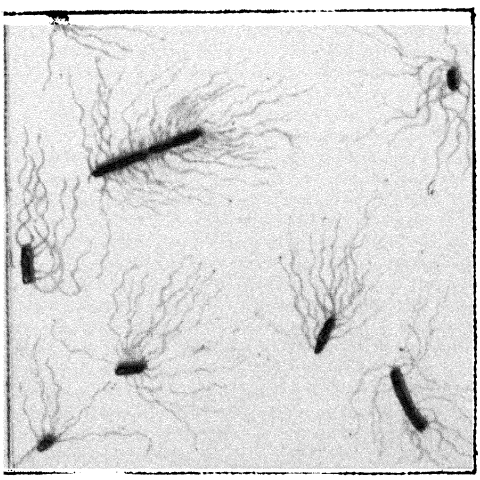


FIG. 15.

PLATE IV.

FIG. 16. Negri bodies in nerve cells in rabies (hippocampus of dog). Alcoholic eosin and methylene-blue. × 1000 diameters.

FIG. 17. Bacillus pestis, showing involution forms, from a salt-agar culture. Thionin-blue. × 1000 diameters.

FIG. 18. Blood film, showing the spirochæte of relapsing fever. Leishman's stain. × 1000 diameters.

FIG. 19. Vibrio choleræ, from a 12-hours' agar culture, showing flagella Rd. Muir's method × 1000 diameters.

FIG. 20. Bacillus tetani, showing spores. Dilute carbol-fuchsin. × 1000 diameters.

PLATE IV.

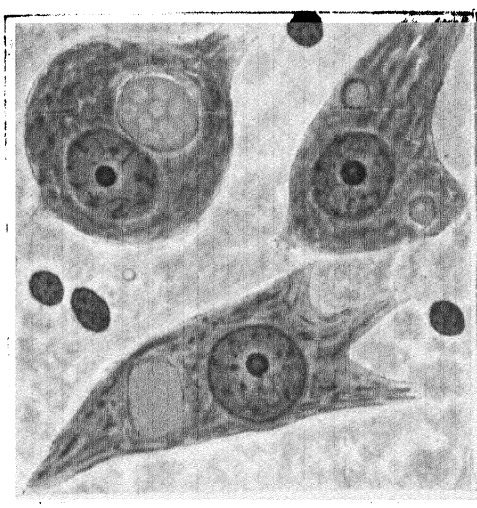


FIG. 16.

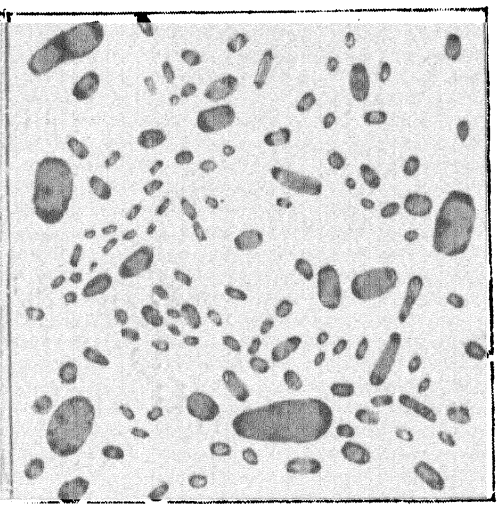


FIG. 17.

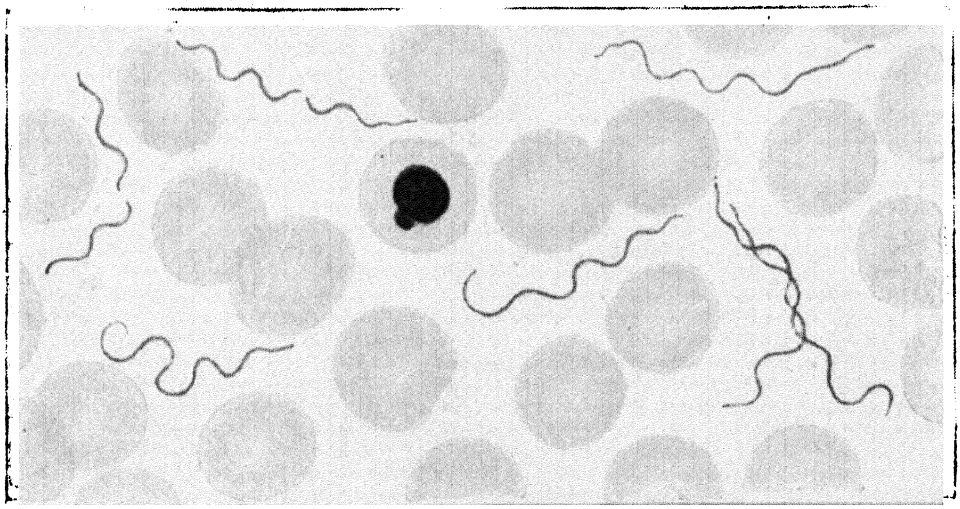


FIG. 18.

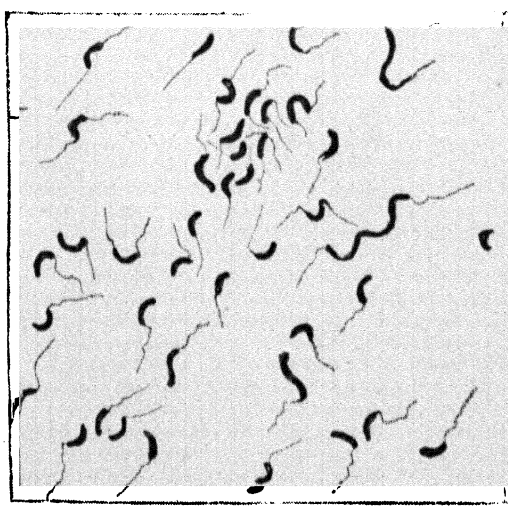


FIG. 19.

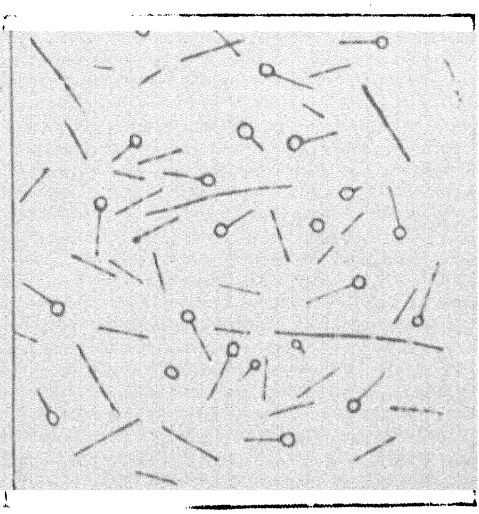


FIG. 20.

PLATE V.

FIG. 21. THE PARASITE OF BENIGN TERTIAN MALARIA.

Cycle I. (Schizogony). Asexual cycle in the human blood.

a. Sporozoite entering red blood corpuscle and forming young trophozoite.

b. Young trophozoite in red blood corpuscle.

c. Young trophozoite in red blood corpuscle, with accumulation pigment.

d. Large pigmented trophozoite.

e. Mature schizont.

f. Commencing segmentation of schizont.

g. Further stage of segmentation.

h. Segmented schizont; formation of merozoites.

i. Disintegration of red blood corpuscle, setting free the merozoites.

j. Young merozoite entering red blood corpuscle.

k. Macrogametocyte, or female sporont.

l. Microgametocyte, or male sporont.

Cycle II. (Sporogony). Sexual cycle in the mosquito.

In the cavity of the stomach of the mosquito.

m. Microgametocyte.

n. Macrogametocyte.

o. Formation of microgametes from the microgametocyte.

p. Free microgamete.

q. Microgamete entering the macrogamete.

r. Zygote or ookinete.

s. Sporocyst.

t. Formation of sporoblasts in the sporocyst.

u. Formation of sporozoites from sporoblasts.

v. Rupture of sporocyst, setting free the sporozoites.

w. Free sporozoites in the body fluid.

x. Accumulation of sporozoites in the salivary gland.

y. Sporozoites passing from gland duct into the blood of man.

FIG. 22. THE PARASITE OF MALIGNANT MALARIA.

a. Young trophozoite entering red blood corpuscle.

b. Do. in red corpuscle.

c. Multiple infection of red corpuscle.

c'. Multiple infection with chromatic stippling in cellular protoplasm; a similar cell is seen lying beneath *a*,—it contains a pigmented trophozoite.

d. Pigmented trophozoite.

e. Segmented schizont, cluster of merozoites.

f. Microgametocyte, "male crescent."

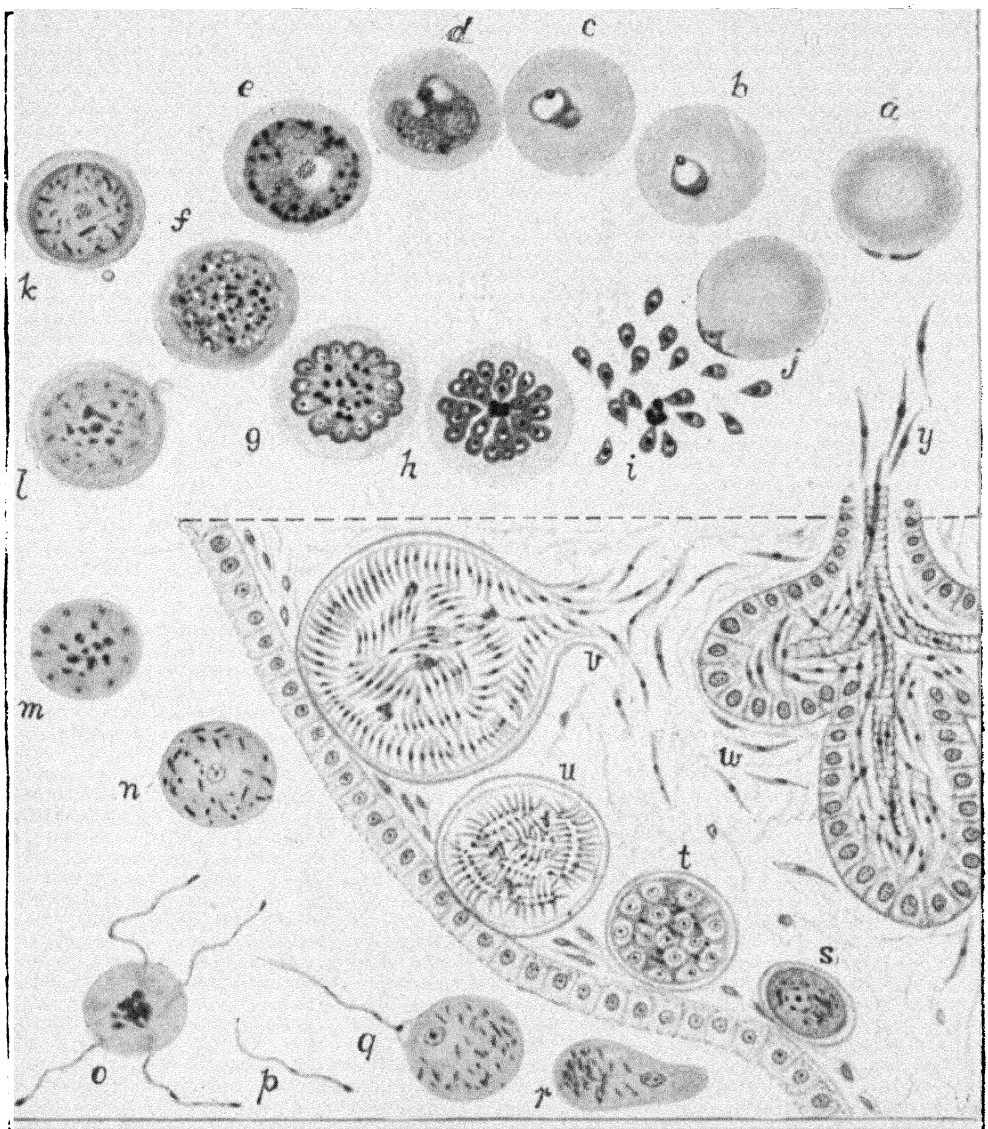
g. Macrogametocyte, "female crescent."

h. Red blood corpuscle with chromatic stippling.

i. Large mono-nucleated phagocyte containing malarial pigment.

PLATE V.

FIG. 21.



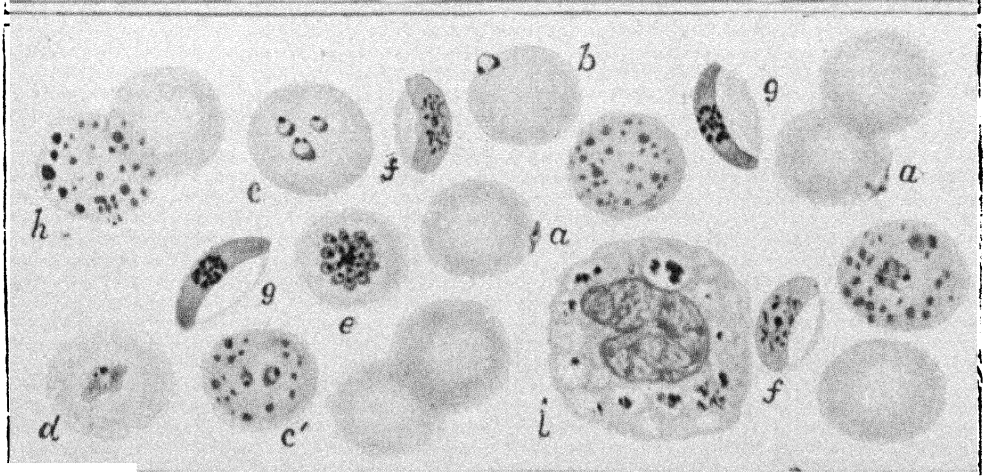


FIG. 22.

PLATE VI.

FIG. 23. Entamœba histolytica in pus, from tropical abscess of liver. Wet-fixed film. Stained by Benda s method.
× 1000 diameters.

FIG. 24. Leishman-Donovan bodies, from the spleen of a case of kala azar. Leishman's stain. × 1000 diameters.

FIG. 25. Blood-film showing Trypanosoma gambiense. Leishman's stain. × 1000 diameters.

PLATE VI.



FIG. 23.



FIG. 24.

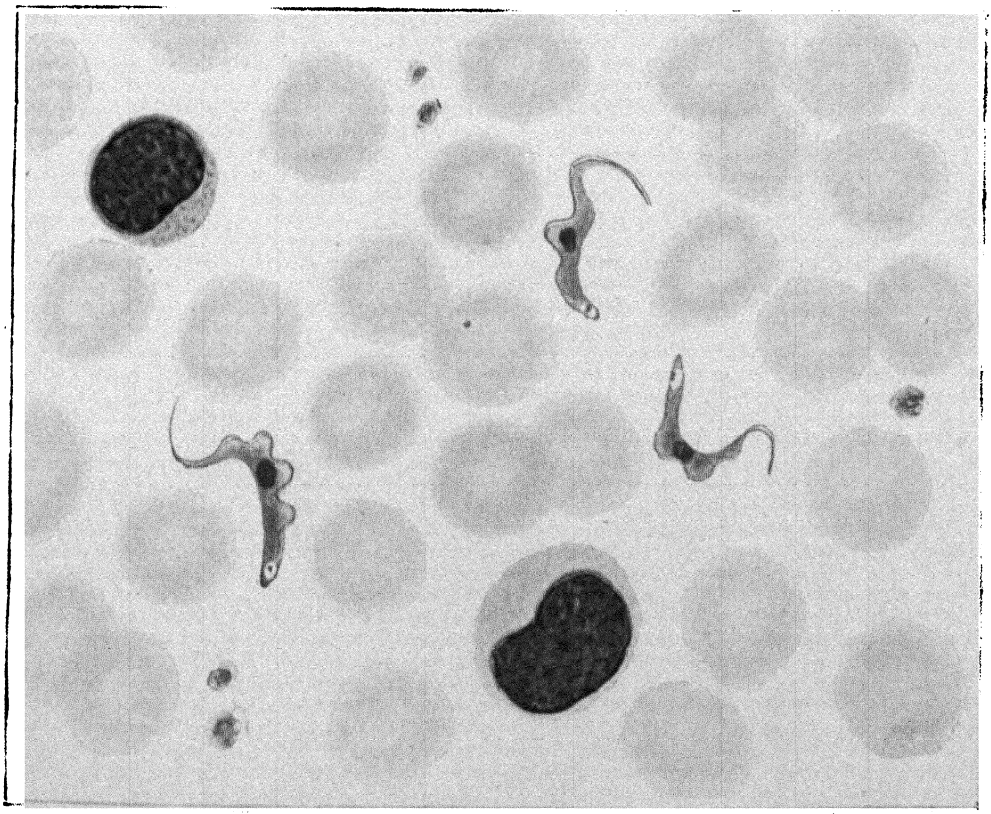


FIG. 25.

MANUAL OF BACTERIOLOGY

CHAPTER I

GENERAL MORPHOLOGY AND BIOLOGY

Introductory.—If the microbes pathogenic for man and the lower animals are considered as a whole, it is found that they belong to a variety of orders, and include primitive types, both of the vegetable and animal kingdoms. The causal agents of the great majority of infective conditions in this and other temperate countries are bacteria, which are accepted as being among the lowest forms of vegetable life; and with these we are chiefly concerned. In addition, however, higher vegetable forms, namely, Hyphomycetes (moulds) and Blastomycetes (yeasts), and also the lowest members of the animal kingdom, the Protozoa, contain many pathogenic species, and thus require consideration in any work concerned with disease-producing micro-organisms. These will accordingly be dealt with in connection with some of the most important diseases produced by them. Classification of unicellular organisms is very difficult, and has been the subject of much controversy; and Haeckel, in order to simplify matters, introduced the term Protista to include the lowest members of both vegetable and animal kingdoms. General agreement amongst systematists is, however, gradually being attained and the principles of classification are becoming stabilised, though there still remain matters of controversy. Further, it is now well recognised that various important and prevalent diseases are due to viruses that are not demonstrable by ordinary methods, owing to their size being beyond the limits of resolution by the microscope, hence called *ultramicroscopic viruses*. Such viruses can be separated from other micro-organisms by their property of filterability through an earthenware filter, and thus they are often known as *filterable*

viruses or *filter-passers*. These terms have, however, only a relative significance, as a given type may pass through or be retained according to the character of the filter, conditions of filtration, etc., and even organisms microscopically visible may pass filters under certain circumstances. The more important diseases produced by filter-passers will also receive consideration.

The bacteria collectively form the class designated *Schizomycetes*. They are unicellular organisms of various forms, devoid of chlorophyll and with imperfectly differentiated nucleus, and multiply by division in one, two, or three directions of space; some are motile, others are non-motile. They are of comparatively simple structure and may be conveniently arranged in two main subdivisions—a lower and simpler, and a higher and better developed.

The *lower forms* or *Eubacteria* are the more numerous, and are minute, relatively undifferentiated masses of protoplasm, which produce similar cells by simple fission. Some are motile, others non-motile. Their minuteness may be judged of by the fact that in one direction at least they usually do not measure more than 1 μ ($\frac{1}{25000}$ inch). These forms can be classified according to their shapes into four main groups—(1) A group in which the shape is globular. The members of this are called *Cocci*. (2) A group in which the shape is that of a straight rod—the proportion of the length to the breadth of the rod varying greatly among the different members. These are called *Bacilli*. (3) A group in which the shape is that of a curved or spiral rod. These are called *Spirilla*. (4) A group of filamentous flexuous organisms showing undulations or true spirality—the *Spirochætæ*. There has been much dispute with regard to the classification of this group of organisms. Many of them are protozoon-like and have been regarded as belonging to the animal kingdom; they are now, however, generally put amongst the bacteria. Again, some place the smaller forms, which are very similar to the spirilla, amongst the lower bacteria, whilst they place the larger forms amongst the higher bacteria. And further, all the spirochætes have been grouped in the American classification as a distinct order of the Schizomycetes co-equal with the Eubacteria (p. 17). A more detailed description of the characters of these groups will be more conveniently taken later (p. 13). In some cases, especially among the bacilli, there may occur under certain circumstances changes in the protoplasm whereby a resting stage or spore is formed.

The *higher forms* show advance on the lower along two lines. (1) On the one hand, they consist of filaments made up of

simple elements such as occur in the lower forms. These filaments may be more or less septate, may be provided with a sheath, and may show branching, either true or false. The minute structure of the elements comprising these filaments is analogous to that of the lower forms. Their size, however, is often somewhat greater. The lower forms sometimes occur in filaments, but here every member of the filament is independent, while in the higher forms there seems to be a certain interdependence among the individual elements. For instance, growth may occur only at one end of a filament, the other forming an attachment to some fixed object. (2) The higher forms, moreover, present this further development, that in certain cases some of the cells may be set apart for the reproduction of new individuals (p. 5).

The relations of the bacteria to the animal kingdom on the one hand and to the vegetable on the other constitute a difficult question. It is best to think of there being a group of small, unicellular organisms, which may be survivals of the most primitive forms of life before differentiation into animal and vegetable types had occurred and in some cases even before in an individual cell nucleus had been differentiated from cytoplasm. This would include the protozoa, the myxomycetes, the lower algæ, and the bacteria. To the lower algæ the bacteria show many similarities. These algæ are unicellular masses of protoplasm, having generally the same shapes as the bacteria, and largely multiplying by fission. Endogenous sporulation, however, does not occur, nor is motility necessarily associated with the possession of flagella. Also their protoplasm differs from that of the bacteria in containing chlorophyll and another blue-green pigment called phycocyan. From the morphological resemblances between these algæ and the bacteria, and from the fact that fission plays a predominant part in the multiplication of both, they were formerly grouped together in one class as the Schizophyta or splitting plants. And of the two divisions forming these Schizophyta the splitting algæ were denominated the Schizophyceæ, while the bacteria or splitting fungi were called the Schizomycetes.

General Morphology of the Bacteria

The Structure of the Bacterial Cell.—When examined under the microscope, in their natural condition, *e.g.* in water, bacteria appear merely as colourless refractile bodies of the different shapes named. Spore formation and motility, when these exist, can also be observed, but little else can be made out. For their proper investigation advantage is always taken of their affinities for various dyes, especially those which are usually chosen as good stains for the nuclei of animal cells. Certain points have thus been determined. The bacterial cell consists

of a sharply contoured mass of protoplasm which reacts to basic aniline dyes like the nucleus of an animal cell. A healthy bacterium when thus stained presents the appearance of a finely granular or almost homogeneous structure. The protoplasm is surrounded by an envelope which can in some cases be demonstrated by overstaining a specimen with a strong aniline dye, when it will appear as a halo round the bacterium. This envelope may sometimes be seen to be of considerable thickness. Its innermost layer is probably of a denser consistence, and sharply contours the contained protoplasm, giving the latter the appearance of being surrounded by a membrane. It is only, however, in some of the higher forms that a definite sheath occurs. Sometimes the outer margin of the envelope is sharply defined, in which case the bacterium appears to have a distinct capsule, and is known as a capsulated bacterium (*vide* Fig. 1, *h*; and Fig. 58). The cohesion of bacteria into masses depends largely on the character of the envelope. If the latter is glutinous, then a large mass of the same species may occur, formed of individual bacteria embedded in what appears to be a mass of jelly; this is known as a *zooglœa* mass. On the other hand, if the envelope has not this cohesive property the separation of individuals may easily take place, especially in a fluid medium in which they may float entirely free from one another. Many of the higher bacteria possess a sheath which has a much more definite structure than is found among the lower forms. It resists external influences, possesses elasticity, and serves to bind the elements of the organism together. In certain forms the sheath contains granules of iron oxide or other substances.

Reproduction among the Lower Bacteria.—When a bacterial cell is placed in favourable surroundings, it multiplies by simple fission. In the process a constriction appears in the middle and a transverse unstained line develops across the protoplasm at that point. The process goes on till two individuals can be recognised, which may remain for a time attached to one another, or become separate, according to the character of the envelope, as already explained. In most bacteria growth and multiplication go on with great rapidity. A bacterium may reach maturity and divide in from twenty minutes to half an hour. If division takes place only every hour, from one individual after twenty-four hours 17,000,000 similar individuals will be produced. As shown by the results of artificial cultivation, others, such as the tubercle bacillus, multiply much more slowly. In some cases the bacterial cell enlarges before division, in others the cell divides and each element then expands to its

adult size. If, in the latter alternative, multiplication is proceeding rapidly, great variation in the size of the individuals may be observed, and this may give rise to anomalous appearances. Amongst the spirochætes longitudinal as well as transverse division has been described, though this is disputed.

From investigations by Graham-Smith and others, it appears that the consistence of the envelope may have an importance in modifying the naked-eye and low-power appearances presented by bacterial colonies, which constitute a feature in the identification of species. Graham-Smith has differentiated four groups—a "loop-forming," in which the envelope is so tough that, after division, rupture but rarely occurs (B. anthracis); a "folding" group, in which the envelope is so flexible and extensile that the members of a chain can be folded on one another as successive divisions take place (B. pestis); a "snapping" group, in which partial rupture of the envelope occurs on division (B. diphtheriæ); and a "slipping" group, where the envelope readily breaks, and successively developed bacteria slip past each other (V. choleræ).

When bacteria are placed in unfavourable conditions as regards food, etc., growth and multiplication take place with difficulty. In the great majority of cases this is evidenced by changes in the appearances of the protoplasm. Instead of its maintaining the regularity of shape seen in healthy bacteria, various aberrant appearances are presented. This occurs especially in the rod-shaped varieties, where flask-shaped or dumb-bell-shaped individuals may be seen. The regularity in structure and size is quite lost. The appearance of the protoplasm also is often altered. Instead of, as formerly, staining well, it does not stain readily, and may have a uniformly pale homogeneous appearance, while in an old culture only a small proportion of the bacteria may stain at all. Sometimes, on the other hand, a degenerated bacterium contains intensely stained granules or globules which may be of large size. Such aberrant and degenerate appearances are referred to as *involution forms* (Fig. 1, i^1, i^2). That these forms really betoken degenerative changes is shown by the fact that, on their being again transferred to favourable conditions, only slight growth at first takes place. Many individuals have undoubtedly died, and the remainder which live and develop into typical forms may sometimes have lost some of their properties.

Reproduction among the Higher Bacteria.—Most of the higher bacteria consist of thread-like structures more or less septate and often surrounded by a sheath. The organism is frequently attached at one end to some object or to another individual. It grows to a certain length and then at the free end certain cells, called conidia, are cast off from which new individuals are formed. These conidia

may be formed by a division taking place in the terminal element of the filament such as has occurred in the growth of the latter. In some cases, however, division takes place in three dimensions of space. The conidia have a free existence for a certain time before becoming attached, and in this stage are sometimes motile. They are usually oval or rod-like, sometimes pyriform. They do not possess any special powers of resistance.

Spore Formation.—In certain species of the lower bacteria, under certain circumstances, changes take place in the protoplasm which result in the formation of bodies called spores, to which the vital activities of the original bacteria are transferred. Spore formation occurs chiefly among the bacilli and in some spirilla. Its commencement in a bacterium is indicated by the appearance in the protoplasm of a minute highly refractile granule unstained by the ordinary methods. This increases in size, and assumes a round, oval, or short rod-shaped form, always shorter but often broader than the original bacterium. In the process of spore formation the rest of the bacterial protoplasm may remain unchanged in appearance and staining power for a considerable time (*e.g.* B. tetani), or, on the other hand, it may soon lose its power of staining and ultimately disappear, leaving the spore in the remains of the envelope (*e.g.* B. anthracis). This method of spore formation is called *endogenous*, and the spores are known as *endospores*. Bacterial spores are always non-motile. The spore may appear in the centre of the bacterium, or it may be at one extremity, or a short distance from one extremity (Fig. 1, *s*). In structure the spore consists of a mass of protoplasm surrounded by a dense membrane or capsule. This can be demonstrated by methods which will be described, the underlying principle of which is the prolonged application of a powerful stain. The all-important property of a bacterial spore is its high degree of resistance to external influences such as heat, drying, chemical agents, etc. Such resistance has been ascribed in certain instances to the capsule. Koch, for instance, in one series of experiments, found that while the Bacillus anthracis in the unspored form was killed by a two minutes' exposure to 1 per cent. carbolic acid, spores of the same organism resisted an exposure of from one to fifteen days.

When a spore is placed in suitable surroundings for growth, it again assumes the original bacillary form. The capsule may dehisce either longitudinally, or terminally, or transversely. In the last case the dehiscence may be partial, and the new individual may remain for a time attached by its

ends to the hinged spore-case, or the dehiscence may be complete and the bacillus grow with a cap at each end consisting of half the spore-case. Sometimes the spore-case does not dehisce, but is simply absorbed by the developing bacterium.

It is important to note that, in the bacteria, spore formation is rarely, if ever, to be considered as a method of multiplication. In at least the great majority of cases only one spore is formed from one bacterium, and only one bacterium in the first instance from one spore. Sporulation is to be looked upon as a *resting stage* of a bacterium, and is to be contrasted with the stage when active multiplication takes place. The latter is usually referred to as the *vegetative stage* of the bacterium. Regarding the signification of spore formation in bacteria, there has been some difference of opinion. According to one view, it may be regarded as representing the highest stage in the vital activity of a bacterium. There is thus an alternation between the vegetative and spore stage, the occurrence of the latter being necessary to the maintenance of the species in its greatest vitality. Such a rejuvenescence, as it were, through sporulation, is known in many algæ. In support of this view there are certain facts. In many cases, for instance, spore formation only occurs at temperatures specially favourable for growth and multiplication. There is often a temperature below which, while vegetative growth still takes place, sporulation will not occur ; and in the case of B. anthracis, if the organism be kept at a temperature above the limit at which it grows best, not only are no spores formed, but the strain may lose the power of sporulation. Furthermore, in the case of bacteria preferring the presence of oxygen for their growth, an abundant supply of this gas may favour sporulation. It is probable that even among bacteria preferring the absence of oxygen for vegetative growth, the presence of this gas favours sporulation. The second view with regard to sporulation is that a bacterium only forms a spore when its surroundings, especially its food supply, become unfavourable for vegetative growth ; it then remains in this condition until it is placed in more suitable surroundings. Such an occurrence would be analogous to the *encystment* which occurs under similar conditions in many of the protozoa. Often sporulation can be prevented from taking place for an indefinite time if a bacterium is constantly supplied with fresh food (the other conditions of life being equal). The presence of substances produced by the bacteria themselves plays, however, a more important part in making the surroundings unfavourable than the mere exhaustion of the food supply. A living spore

will always develop into a vegetative form if placed in a fresh food supply. With regard to the rapid formation of spores when the conditions are favourable for vegetative growth, it must be borne in mind that in such circumstances the conditions may really very quickly become unfavourable for a continuance of growth, since not only will the food supply around the growing bacteria be rapidly exhausted, but the formation of effete and inimical matter will be all the more rapid.

We must note that the usually applied tests of a body developed within a bacterium being a spore depend on (1) its staining reaction, namely, resistance to ordinary staining fluids, but capacity of being stained by the special methods devised for the purpose (*vide* p. 110); (2) the fact that the bacterium containing the spore has higher powers of resistance against inimical conditions than a vegetative form. It is important to bear these tests in mind, as, in some of the smaller bacteria especially, it is very difficult to say whether they spore or not. There may appear in such organisms small unstained spots, the significance of which is very difficult to determine; in every case the test of resistance must be made.

The Question of Arthrosporous Bacteria.—The view has been put forward that among certain organisms, *e g* some streptococci, certain individuals may, without endogenous sporulation, take on a resting stage. These become swollen, stain well with ordinary stains, and they are stated to have higher power of resistance than the other forms; further, when vegetative life again occurs, it is from them that multiplication is said to take place. From the fact that there is no new formation within the protoplasm, but that it is the whole of the latter which participates in the change, these individuals have been called *arthrospores*. The existence of such special individuals amongst the lower bacteria is extremely problematical. They have no distinct capsule, and they present no special staining reactions, nor any microscopic features by which they can be certainly recognised, while their alleged increased powers of resistance are very doubtful. All the phenomena noted can be explained by the undoubted fact that in an ordinary growth there is very great variation among the individual organisms in their powers of resistance to external conditions.

Motility.—As has been stated, many bacteria are motile. Motility can be studied by means of hanging-drop preparations (*vide* p. 96). The movements are of a darting, rolling, or vibratile character. The degree of motility depends on the species, the temperature, the age of the growth, and on the medium in which the bacteria are growing. Sometimes the movement is most active just after the cell has multiplied, sometimes it goes on all through the life of the bacterium,

sometimes it ceases when sporulation is about to occur. Motility is associated with the possession of fine wavy thread-like appendages called flagella, which for their demonstration require the application of special staining methods (*vide* Fig. 1, *q*, and Fig. 107). They have been shown to occur in many bacilli and spirilla, but only in a few species of cocci. They vary in length, but may be several times the length of the bacterium, and may be at one or both extremities or all round. When terminal they may occur singly or there may be several; in some spirilla a tuft of terminal flagella is present. Sometimes complicated spiral tresses of free flagella are found in bacterial cultures; the development of these is difficult to explain. The nature of flagella has been much disputed. Some have held that, unlike what occurs in many algæ, they are not actual prolongations of the bacterial protoplasm, but merely appendages of the envelope, and have doubted whether they are really organs of locomotion. There is now, however, little doubt that they belong to the protoplasm. It must be recognised, however, that not all cases of motility among the bacteria are dependent on the possession of flagella, for amongst the spirochætes the movements, which are of various kinds, are due to contractions of the protoplasm of the cell itself. Among most of the higher bacteria also, motility is of similar nature.

The Minuter Structure of the Bacterial Protoplasm.—Many attempts have been made to obtain deeper information as to the structure of the bacterial cell, especially with reference to the existence of a differentiation into nucleus and cytoplasm and as to the intimate phenomena of division. Observations bearing on such points can only be made on certain large species, but even with these the minuteness of the cells makes the interpretation of the appearances seen most difficult. While bacterial protoplasm generally exhibits a selective action for nuclear aniline dyes, the material thus picked out appears in certain bacteria not to be uniformly distributed through the cell, but to be deposited in certain parts, and controversy has turned on the interpretation of such appearances. Two main views have been held by different schools. Some consider that the bacterial cell contains a formed nucleus and a cytoplasm; at the same time it is questioned whether all the material giving the reaction of a nucleus is really part of such a central structure and not merely stored material. A modification of this view looks on the nucleus as an extended thread lying in the protoplasm—in some bacillary types having a spiral or zigzag appearance. The other view is that the bacterial cell represents a vital unit in which differentiation into nucleus and cytoplasm has not yet occurred, or has become lost, and where the two main elements of higher cells are intermingled with one another, the homologue of the cytoplasm being present in a close meshwork of nuclear material. With regard to the behaviour of the cell in division, amongst those who hold the

former view some have figured appearances in the supposed nucleus which suggest the occurrence of mitosis, but such an interpretation is not generally accepted. It is to be noted, however, that it is only in certain of the larger forms that supposed nuclear structure has been found, and that even then it may have another significance ; in the vast majority of bacteria the nucleus is of the diffuse type and cannot be satisfactorily differentiated. We may add that bacteria have been found to contain a considerable proportion of nucleoprotein. (A point of interest is that in the case of the tubercle bacillus the basic constituents correspond with those in animal and not in vegetable tissues.)

Bütschli, from a study of some large sulphur-containing forms, concluded that the greater part of the bacterial cell may correspond to a nucleus, and that this is surrounded by a thin layer of protoplasm which in the smaller bacteria escapes notice, unless when it can be made out at the ends of the cells. Fischer, it may be said, looked on the appearances seen in Butschli's preparations as due to plasmolysis (*vide infra*).

Before bacteria exceeding, say, 1 to 1·5 μ in thickness were known, appearances analogous to those described had been recognised among the smaller forms, even when stained by ordinary methods. Occasionally irregular, deeply staining granules had been observed in the protoplasm, often, when they occurred in a bacillus, giving the latter the appearance of a short chain of minute cocci. These were called metachromatic granules from the fact that by appropriate procedure they could be stained with one dye, while the rest of the bacterial cell could be made to take on another colour. Such an appearance is well known as occurring in the diphtheria bacillus, especially when stained by Neisser's method (p. 110). In certain bacteria, for example the plague bacillus, the stainable substance is arranged at each pole, leaving an unstained spot between—the so-called "polar staining." At present it is impossible to interpret the significance of such appearances. They are present in certain bacteria under all circumstances, sometimes they are associated with growth in particular surroundings. In some species the presence of granules is an indication of lowered vitality.

Whatever the composition and relationships of the essential parts of the bacterial protoplasm may be, there is, as has been said, reason for believing that even in the lower forms reserve material exists. This may consist of fat, glycogen, and other substances, amongst which may be mentioned volutin, as described by A. Meyer, a substance probably of protein nature characterised by solubility in water, alkalis and acids, and by insolubility in alcohol.

In perfectly healthy and young bacteria, appearances of granule formation and of vacuolation may be artificially produced by physical means from the occurrence of what is known as *plasmolysis*. To speak generally, when a mass of protoplasm surrounded by an envelope of a colloidal nature is placed in a solution containing salts in greater concentration than that in which it has previously been living, then by a process of osmosis the water held in the protoplasm passes out through the membrane, and artificial appearances may result. Now, in making a dried film for the microscopic examination of bacteria, the conditions necessary for the occurrence of this process may be produced and the appearance, in certain cases, of polar granules may thus be brought about. Plasmolysis

in bacteria has been extensively investigated, and has been found to occur in some species more readily than in others. Furthermore, it is often more readily observed in old or otherwise enfeebled cultures.

The Chemical Composition of Bacteria.—The chemical structure of bacterial protoplasm has been investigated both by micro- and macro-chemical methods—the former being chiefly applicable to the larger forms. With iodine, granules staining brownish-red or blue have been observed, and these are looked on as composed of substances allied to glycogen and starch respectively. Similarly, reactions with osmic acid, scharlach and similar dyes, have pointed to the presence of fats. While macro-chemical investigation has not thrown much light on the occurrence of carbohydrates, cellulose is said to be obtainable from certain bacteria. Bodies giving the reactions of fats have been isolated in bulk and have received much attention in the case of the tubercle bacillus group, whose special staining characteristics are probably due to bodies of this class. The substances mentioned are to be looked upon as reserve material or metabolic products in the life of the bacterial cell; but substances of a protein nature have also been derived from bacterial protoplasm, and these are probably more intimately related to the vital structures of the organism. Chemically they are allied to, or are identical with, similar substances found in plant and animal tissues, for example, albumins, globulins, and phosphorised substances such as nucleins and nucleic acid. There is also evidence that in the bacteria, as in the higher cells, lipoidal bodies are intimately associated with the protein elements. Further, various mineral salts, especially those of sodium, potassium, and magnesium, are constituents of bacterial protoplasm. All the constituents show great variations, dependent not only on the species under investigation, but also on the composition of the culture media, on the temperature of growth, and on the age of the culture.

Many species of bacteria, when growing in masses, are brilliantly coloured, though few bacteria associated with the production of disease give rise to pigments. In some of the higher bacteria a pigment named bacterio-purpurin has been observed in the protoplasm, and similar intracellular pigments probably occur in some of the larger forms of the lower bacteria and may occur in the smaller; but it is usually impossible to determine whether the pigment occurs inside or outside the protoplasm. In many cases, for the free production of pigment abundant oxygen supply is necessary; but sometimes, as in

the case of Spirillum rubrum, the pigment is best formed in the absence of oxygen. Sometimes the faculty of forming it may be lost by an organism for a time, if not permanently, by the conditions of its growth being altered. Thus, for example, if the B. pyocyaneus be exposed to the temperature of 42° C. for a certain time, it loses its power of producing its bluish pigment. Pigments formed by bacteria often diffuse out into, and colour, the medium for a considerable distance around.

Comparatively little is known of the nature of bacterial pigments. Zopf, however, has found that many of them belong to a group of colouring matters which occur widely in the vegetable and animal kingdoms, namely, the lipochromes. These lipochromes, which get their name from the colouring matter of animal fat, include the colouring matter in the petals of Ranunculaceæ, the yellow pigments of serum and of the yolks of eggs, and many bacterial pigments. The lipochromes are characterised by their solubility in chloroform, alcohol, ether, and petroleum, and by their giving indigo-blue crystals with strong sulphuric acid, and a green colour with iodine dissolved in potassium iodide. Though crystalline compounds of these have been obtained, their chemical constitution is entirely unknown, and even their percentage composition is disputed

The Classification of Bacteria.—In what we have to say under this heading we shall chiefly confine ourselves to the characters of the pathogenic bacteria. There have been numerous schemes set forth for the classification of bacteria, the fundamental principle running through all of which has been the recognition of the two sub-groups and the type forms mentioned in the opening paragraph above. In the past there has been little agreement among systematists as to the characters on which more detailed classification should be based, and even yet our knowledge of the essential morphology and relations of the bacteria is too limited for an exact classification on a strictly biological basis. Identification of species is dependent, not only on the morphology of individual organisms and that of growth in culture media, but also on physiological and biochemical characters, pathogenicity to animals under experimental conditions, and in some cases on delicate serum reactions. Recently an extensive scheme of classification has been put forward by the Society of American Bacteriologists, and this will be referred to below after a simple general account has been given.

The division into lower and higher bacteria is recognised by all, though, as in every other classification, transitional forms have to be accounted for. In subdividing the bacteria further, the forms they present constitute at present a practicable basis of classification. The lower bacteria thus naturally fall into the

four morphological groups mentioned, the (1) cocci, (2) bacilli, (3) spirilla, and (4) spirochætes, though the higher are more difficult to deal with. Subsidiary, though important, points in the further subdivision are the planes in which fission takes place (*e.g.* among the cocci), the presence or absence of spores, etc. The recognition of actual species is often a matter of great difficulty. The points to be observed in this will be discussed in connection with the individual organisms.

I. The Lower Bacteria.[1]—These, as we have seen, are minute unicellular masses of protoplasm surrounded by an envelope, the total vital capacities of a species being represented in every cell. They present four distinct type forms just mentioned. Endogenous sporulation may occur; they may also be motile.

1. *The Cocci.*—In this group the cells range in different species from 0·5 μ to 2 μ in diameter, but most measure about 1 μ. Before division they may increase in size in all directions. The main groups are usually classified according to the method of division. If the cells divide only in one axis, and through the consistency of their envelopes remain attached, then a chain of cocci will be formed. A group in which this occurs is known as a *streptococcus*. If division takes place irregularly, the resultant mass may be compared to a bunch of grapes, and the group is often called a *staphylococcus*. Division may take place in two axes at right angles to one another, in which case cocci adherent to each other in plates of four (called *tetrads*) or multiples of four may be found, the former number being the more frequent. To all these forms the word *micrococcus* may be generally applied. The individuals in a growth of micrococci often show a tendency to remain united in twos. These are spoken of as *diplococci*, but this is not a distinctive character, since every coccus as a result of division becomes a diplococcus, though in some species the tendency to remain in pairs is well marked. The adhesion of cocci to one another depends on the character of the capsule. Often this has a well-marked outer limit (*Micrococcus tetragenus*), sometimes it is of great extent, its diameter being many times that of the coccus (*Streptococcus mesenteroides*). In none of the cocci have endogenous spores been certainly observed. Usually included in this group are coccus-like organisms which divide in three axes at right angles to one another. These are referred to as *sarcinæ*. If the cells are lying singly they are round, but usually they are seen in cubes of eight with the sides which

[1] For the illustration of this and the succeeding systematic paragraphs, *vide* Fig. 1.

are in contact slightly flattened. Large numbers of such cubes may be lying together. The sarcinæ are, as a rule, rather larger than the other members of the group. Most of the cocci are non-motile, but a few motile species possessing flagella have been described; possibly, however, these are more of the nature of short bacilli—*cocco-bacilli*.

2. *Bacilli*.—These consist of long or short cylindrical cells, with rounded or sharply rectangular ends, usually not more than 1 μ broad, but varying very greatly in length. They may be motile or non-motile. Where flagella occur, these may be distributed all round the organism—*peritrichous*, or only at one or both of the poles—*terminal*. Several species are provided with sharply-marked capsules (*e.g.* pneumo-bacillus). In many species endogenous sporulation occurs. The spores may be central, terminal, or subterminal, round, oval, or spindle-shaped. There is no doubt that among the bacilli in certain cases, *e.g.* in B. diphtheriæ and B. tuberculosis, the phenomenon of true branching may occur. Such instances form a connecting link between the bacilli and the higher bacteria, *e.g.* streptothrices.

3. *Spirilla*.—These consist of curved rods or cylindrical non-flexuous cells more or less spiral or wavy. The unit is usually a short curved rod or *vibrio* (often referred to as of a "comma" shape). When two or more of the latter occur, as they often do, end to end with their curves alternating, then a wavy or spiral thread results. An example of this is the cholera microbe (Fig. 1, *p*). The motile spirilla possess terminal flagella. Of the latter there may be one or two, or a bunch containing as many as twenty, at one or both poles (Fig. 1, q^4). Division by transverse fission takes place as among the bacilli.

4. *Spirochætæ*.—These are elongated cells which show undulation or regular spirality. As a rule they are thin in proportion to their length, and some of the smaller forms are of extreme tenuity. Motility is due to contraction of the protoplasm and may be of a lashing, wave-like, oscillatory, or corkscrew-like character. Some are tapered at their extremities to a flagellum-like structure. The larger forms reach a considerable size and a relatively great length, and some of them have a spirally-twisted crest running along the whole length of the cell. They multiply by transverse fission, but longitudinal division has been described in some of them. The pathogenic species belong to different genera—*Treponema* (organisms of syphilis and yaws, etc.), and *Leptospira* (organisms of yellow fever and infective jaundice) (*vide* p. 562).

II. The Higher Bacteria.—These show advance on the lower

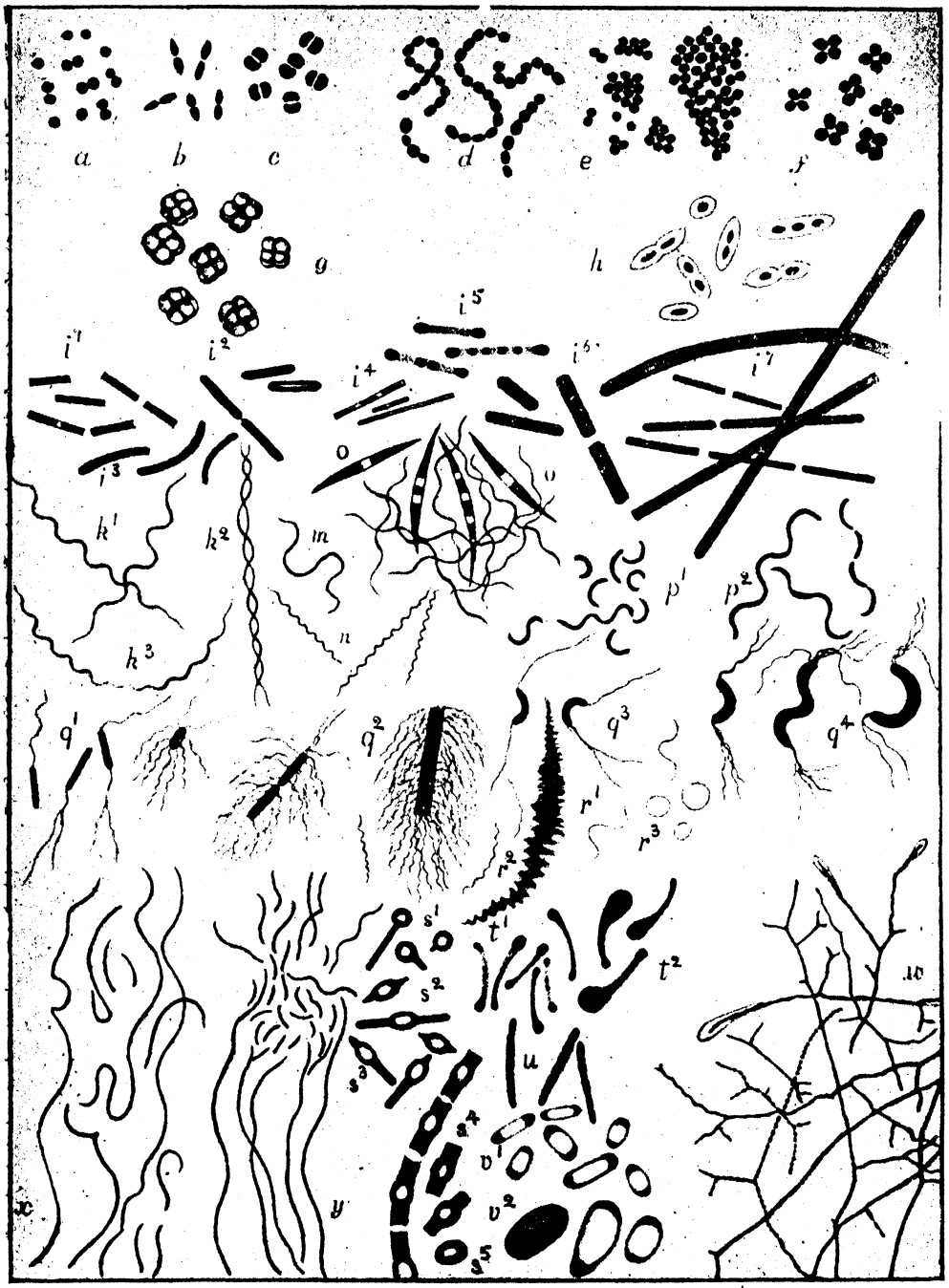


FIG. 1.—*a–h*. Different types of cocci. *a*. Single round cocci and simple diplococcal forms. *b*. Lancet-shaped cocci (pneumococcus). *c*. Bean-shaped cocci (gonococcus). *d*. Streptococci. *e*. Staphylococci. *f*. Tetrads (Micrococcus tetragenus). *g*. Sarcina forms. *h*. Capsulated cocci. i^1–i^7. Bacilli. i^1–i^3. Ordinary types of different shapes. i^4, i^5. Bacilli with granular or vacuolated protoplasm. i^6, i^7. Large forms. k^1–*n*. Spirochætes. k^1. Spirochæte with open turns (Spironema refringens). k^2. Possible longitudinal splitting of spirochæte. k^3. Two individuals separating. *m*. Spirochæte with irregular turns. *n*. Spirochæte with close turns (Treponema pallidum). *o*. Mixed type of fusiform bacilli and spirochætes (see Chapter XXI). *p*. Spirilla. p^1. Comma type. p^2. Spirillary type. *q*. Different types of flagellum formation. q^1. Terminal flagella. q^2. Peritrichous arrangement. q^3. Flagella on spirillum. q^4. Large flagellated spirillum. r^1. Wreathed mass of flagella. r^2. Detached flagellum. r^3. Detached flagella assuming ring form. *s*. Types of sporulation. s^1. Terminal. s^2, s^4. Central. s^3. Subterminal. s^5. Detached spores. t^1, t^2. Involution forms (B. diphtheriæ). *u*. Diphtheroid bacillus. v^1–v^2. Involution forms (B. pestis). *w*. Streptothrix actinomyces. *x*. Leptothrix innominata. *y*. Thiothrix tenuis.

in consisting of definite filaments branched or unbranched. In most cases the filaments at more or less regular intervals are cut by septa into short rod-shaped or curved elements. Such elements are more or less interdependent on one another, and special staining methods are often necessary to demonstrate the septa which demarcate the individuals of a filament. There is further often a definite membrane or sheath common to all the elements in a filament. Not only, however, is there this close organic relationship between the elements of the higher bacteria, but there is also interdependence of function ; for example, one end of a filament is frequently concerned merely in attaching the organism to some other object. The greatest advance, however, consists in the setting apart among most of the higher bacteria of the free terminations of the filaments for the formation of *conidia* ; groups of such conidia may be formed by division in three directions of space, for the production of new individuals, as has been described (p. 5). There is still diversity of opinion as to the classification of the higher bacteria, but the following may be given as a brief summary of the main orders : (*a*) The *Chlamydobacteria* or *Trichobacteria* are filamentous forms, occurring mainly in water, and are usually sheathed ; the sheath is often impregnated with iron oxide. They are often sessile, and the attached ends thinner than the distal. The leptothrix organisms which are common commensals of the mouth, belong to this order. Conidia formation is often met with in the free ends of the filaments ; the conidia may be motile or non-motile. True branching does not occur, but false branching is sometimes met with, due to a terminal cell becoming laterally displaced, *e.g.* in cladothrix. (*b*) A further development occurs in the *Streptothrices*, to which the Streptothrix actinomyces and other pathogenic species belong. Here the organism consists of a felted mass of non-septate filaments in which true dichotomous branching occurs. Under certain circumstances threads grow out and produce chains of coccus-like bodies or conidia, from which new individuals can be reproduced. These conidia are sometimes spoken of as spores, but they have not the same staining reactions nor resisting powers of so high a degree as the endospores of bacteria. Sometimes, too, the protoplasm of the filaments breaks up into bacillus-like elements which may also have the capacity of originating new individuals. In the Streptothrix actinomyces a club-shaped swelling of the membrane at the end of the filament may occur ; this is probably a product of a degenerative change, or possibly of defensive nature. The streptothrix group may be regarded as a link between the

bacteria on the one hand and the lower fungi on the other. Like the latter the streptothrix forms show the felted mass of branching filaments which is usually called a mycelium, whilst the breaking-up of the protoplasm into coccus- and bacillus-like forms links it to the other bacteria. The streptothrix organisms are put by some writers amongst the chlamydobacteria. (*c*) The *Thiobacteria* or sulphur bacteria are characterised by the presence of sulphur granules in their protoplasm: bacteriopurpurin also may be present. Some of them are sheathed and attached and form conidia, *e.g.* Thiothrix; others again are elongated, sheathless, and show an undulatory motion due to the contraction of the protoplasm, *e.g.* Beggiatoa.

New Classification and Nomenclature of the Society of American Bacteriologists. — In recent years, owing to the unsatisfactory state of bacterial classification and nomenclature, an attempt has been made by the Society of American Bacteriologists to develop a new system of classification in strict accordance with that adopted in other branches of biological study. The classification advocated seems sufficiently elastic to provide for future modification as our knowledge of the relationships of the bacteria is added to. This system introduces a new nomenclature and eliminates the trinomial and clumsy designations that have crept into bacteriology, but it seems doubtful whether new names should be applied to certain well-known pathogenic organisms which already possess a convenient designation recognised by long usage. Such terms as gonococcus, meningococcus, Bacillus typhosus, etc., are likely to maintain their traditional use in preference even to designations that clearly indicate the approved biological genus and species of the organism. The new classification, however, serves a most helpful purpose in its orderly grouping together of biologically allied species.

The bacteria are collectively designated as the class of schizomycetes which is defined in a general way as follows: minute, unicellular organisms, chlorophyll-free, usually colourless; multiplying by division in one, two, or three directions of space; cells—spherical, cylindrical, comma-shaped, spiral, filamentous; or united into filamentous, flat, or cubical aggregates; filamentous aggregates often surrounded by a common sheath; plasma—homogeneous, without a morphologically differentiated nucleus; reproducing by simple fission; in some species resting bodies in the form of endospores and conidia; cells in certain species motile and possessing flagella.

The class of Schizomycetes is divided into the following six orders:—

1. *Eubacteriales* (true bacteria).—Undifferentiated simple forms; spherical, rod-shaped, or spiral; no true filaments; rarely branched; some motile due to flagella; non-flexuous; multiplying by transverse fission; some produce endospores; no conidia.

2. *Spirochætales*.—Specialised and differentiated forms, "protozoa-like"; usually relatively slender, flexuous spirals; multiplying by longitudinal and transverse fission.

3 *Chlamydobacteriales.*—Including the " iron bacteria," which are " plant-like " sheathed organisms, the sheath containing iron oxide.

4. *Thiobacteriales.*—Including the " sulphur bacteria," which are " plant-like " and produce sulphur or bacterio-purpurin granules in their protoplasm.

5. *Actinomycetales.*—Elongated or filamentous " mould-like " organisms with a tendency to branch and produce mycelium (Str. actinomyces) ; often showing " club-shaped " formations ; without spores but producing conidia in some species ; mostly Gram-positive ; non-motile ; some are parasites of animals and plants ; aerobic but some anaerobic ; growth slow.

6. *Myxobacteriales*—Including the myxobacteria ; organisms exhibiting a pseudo-plasmodial and a resistant cyst-forming phase, " slime-mould " like in nature.

It may be noted that in this scheme the first two orders constitute the *lower bacteria* as above described, whilst the other four orders make up the *higher bacteria.* The organisms pathogenic to the human subject belong to the orders 1, 2, and 5. For details as to further divisions, reference must be made to the original publication. In describing the individual bacteria, we shall give in italics the names adopted in the American system

General Biology of the Bacteria

There are five prime factors in the growth of bacteria which must be considered, namely, food supply, moisture, relation to gaseous environment, temperature, and light.

Food Supply.—The bacteria are chiefly found living on the complicated organic substances which form the bodies of dead plants and animals, or which are excreted by the latter while they are yet alive. Seeing that, as a general rule, many bacteria grow side by side, the food supply of any particular variety is, relatively to it, altered by the growth of the other varieties present. It is thus impossible to imitate the complexity of the natural food environment of any species. The artificial media used in bacteriological work may therefore be poor substitutes for the natural supply. In certain cases, however, the conditions under which we grow cultures may be better than the natural conditions. For while one of two species of bacteria growing side by side may favour the growth of the other, it may also in certain cases hinder it, and therefore when the latter is grown alone it may grow better. Most bacteria seem to form products which are unfavourable to their own vitality, for, when a species is sown on a mass of artificial food medium, it soon ceases to grow, even before the food supply is exhausted. In supplying artificial food for bacterial growth, the general principle ought to be to imitate as nearly as possible the natural surroundings, though it is found that there exists a considerable

adaptability among organisms. With the pathogenic varieties it is usually found expedient to use media derived from the fluids of the animal body, and in cases where bacteria growing on plants are being studied, infusions of the plants on which they grow are frequently used. Some bacteria can exist on inorganic food, but most require organic material to be supplied. Of the latter, some require protein to be present for their proper nourishment, while others can derive their nitrogen from a non-protein such as asparagin. All bacteria require nitrogen to be present in some form, and many require to derive their carbon from carbohydrates. Mineral salts, especially sulphates, chlorides, and phosphates, and even in some cases also salts of iron are necessary. Occasionally special substances are needed to support life. Thus some species, in the protoplasm of which sulphur granules occur, require sulphuretted hydrogen to be present. In nature the latter is usually furnished by other bacteria. Again, the influenza bacillus must, outside the animal body, be provided with hæmoglobin, and for the growth of the gonococcus and the meningococcus serum is an essential constituent of a medium. These requirements in the case of particular organisms will be discussed later. When the food supply of a bacterium fails, it degenerates and dies. The proof of death lies in the fact that when it is transferred to fresh and good food supply it does not multiply. If the bacterium forms spores, it may then survive the want of food for a very long time. It may here be stated that the reaction (H-ion concentration) of the food medium is a matter of great importance. Most bacteria prefer a slightly alkaline medium, and some, *e.g.* the cholera vibrio, will not grow in the presence of the smallest amount of free acid. This is referred to in greater detail later.

Moisture.—The presence of water is necessary for the continued growth of all bacteria. The amount of drying which bacteria in the vegetative stage will resist varies very much in different species. Thus the cholera vibrio is killed by two or three hours' drying, while the Staphylococcus pyogenes aureus will survive ten days' drying, and the Bacillus diphtheriæ still more. In the case of spores the periods are much longer. Anthrax spores will survive drying for several years, but here again moisture enables them to resist longer than when they are quite dry. When organisms have been subjected to such hostile influences, even though they survive, it by no means follows that they retain all their vital properties.

Relation to Gaseous Environment.—The relation of bacteria to the oxygen of the air is such an important factor in the life

of bacteria that it enables a biological division to be made among them. Some bacteria will only live and grow when free oxygen is present. To these the title of *obligatory aerobes* is given. Other bacteria will only grow when no free oxygen is present. These are called *obligatory anaerobes*. In still other bacteria the presence or absence of oxygen is a matter of indifference; such organisms are usually denominated *facultative anaerobes*—they being preferably aerobic but capable of existing without oxygen. An example of an obligatory aerobe is B. subtilis; of an obligatory anaerobe, B. tetani, while the great majority of pathogenic bacteria are facultative anaerobes. Some bacteria flourish best in the presence of a minute trace of oxygen, as is shown by the fact that, in a deep culture, growth is most abundant at a point a short distance below the surface; to such organisms the term *micro-aerophilic* has been applied. According to M'Leod and Gordon, the anaerobic character of organisms, such as B. tetani or B. welchii, depends on the fact that in cultures in the presence of oxygen they form peroxide of hydrogen. Certain aerobic bacteria also form this substance, but in virtue of the catalase which they produce at the same time they are protected from the peroxide, which is thus destroyed before it can accumulate in an amount sufficient to prove harmful. B. tetani and other anaerobes fail to form catalase, and as they are extremely susceptible to peroxide, growth cannot go on in the presence of air. With regard to anaerobes, hydrogen and nitrogen are indifferent gases. Many anaerobes, however, do not flourish well in an atmosphere of carbon dioxide. Very few experiments have been made on the action on bacteria of gas under pressure. A great pressure of carbon dioxide is said to make the B. anthracis lose its power of sporing, but seems to have no effect on its vitality or on that of the B. typhosus; in the case of the B. pyocyaneus, however, such pressure is said to destroy life.

Temperature.—For every species of bacterium there is a temperature at which it grows best. This is called the "optimum temperature." There is also in each case a maximum temperature above which growth does not take place, and a minimum temperature below which growth does not take place. As a general rule the optimum temperature is about the temperature of the natural habitat of the organism. For organisms taking part in the ordinary processes of putrefaction the temperature of warm summer weather (20° to 24° C.) may be taken as the average optimum, while for organisms normally inhabiting animal tissues 35° to 39° C. is a fair

average. The lowest limit of ordinary growth is from 12° to 14° C., and the upper is from 42° to 44° C. In exceptional cases growth may take place as low as 5° C., and as high as 70° C. Some organisms which grow best at a temperature of from 60° to 70° C. have been isolated from dung, the intestinal tract, etc. These have been called *thermophilic* bacteria. It is to be noted that while growth does not take place below or above a certain limit, it by no means follows that death takes place outside such limits. Organisms can resist cooling below their minimum or heating beyond their maximum without being killed. Their vital activity is merely paralysed. Especially is this true of the effect of cold on bacteria. The results of different observers vary; but if we take as an example the cholera vibrio, Koch found that while the minimum temperature of growth was 16° C., a culture might be cooled to −32° C. without being killed. With regard to the upper limit, few ordinary organisms in a spore-free condition will survive a temperature of 57° C., if long enough applied. Many organisms lose some of their properties when grown at unnatural temperatures. Thus many pathogenic organisms lose their virulence if grown above their optimum temperature, and some chromogenic forms, most of which prefer rather low temperatures, lose their capacity of producing pigment, *e.g.* Spirillum rubrum.

Effect of Light.—Much attention has been paid to this factor in the life of bacteria. Direct sunlight is found to have a very inimical effect. It has been found that an exposure of dry anthrax spores for one and a half hours to sunlight kills them. When they are moist, a much longer exposure is necessary. Typhoid bacilli are killed in about one and a half hours, and similar results have been obtained with many other organisms. In such experiments the thickness of the medium surrounding the growth is an important point. Death takes place more readily if the medium is scanty or if the organisms are suspended in water. Any fallacy which might arise from the effect of the heat rays of the sun has been excluded, though light plus heat is more fatal than light alone. In direct sunlight it is chiefly the green, violet, and the ultra-violet rays which are fatal. The last-mentioned rays, however produced, have a powerful bactericidal action. By using a quartz spectrometer with a tungsten arc, Browning and Russ showed that the ultra-violet rays with bactericidal action occupy a position in the spectrum at some distance from the visible rays—from 2960 to nearly 2100 Ångström units. The exact extent varies somewhat in the case of different organisms, but the area of rays in the

spectrum effective against any one organism is comparatively sharply marked off. The bactericidal rays have little penetrating power, being completely absorbed by human skin in a thickness of 10 mm. These observers also found that those, and only those, rays which are bactericidal to the Staphylococcus aureus are absorbed by an emulsion of that organism. Diffuse daylight has also an injurious effect upon bacteria, though it takes a much longer exposure to do serious harm. A powerful electric light is as fatal as sunlight. Here, as with other factors, the results vary very much with the species under observation. Some bacteria, especially occurring on the dead bodies of fresh fish, are phosphorescent; also strains of vibrios isolated from fæces have been found to possess this property in culture media.

Conditions affecting the Movements of Bacteria.—In some cases differences are observed in the behaviour of motile bacteria, contemporaneous with changes in their life-history. Thus, in the case of B. subtilis, movement ceases when sporulation is about to take place. On the other hand, in the bacillus of symptomatic anthrax, movement continues while sporulation is progressing. Under ordinary circumstances motile bacteria appear not to be constantly moving, but occasionally to rest; the movements become more active if the temperature be raised. Most interest, however, attaches to the fact that bacilli may be attracted to certain substances and repelled by others. Schenk, for instance, observed that motile bacteria were attracted to a warm point in a way which did not occur when the bacteria were dead and therefore subject only to physical conditions. Most important observations have been made on the attraction and repulsion exercised on bacteria by chemical agents, which have been denominated respectively *positive* and *negative chemotaxis*. Pfeffer investigated this subject in many unicellular organisms. The method used was to fill with the agent a fine capillary tube, closed at one end, to introduce this into a drop of fluid containing the bacteria under a cover-glass, and to watch the effect through the microscope. The general result was to indicate that motile bacteria may be either attracted or repelled by the fluid in the tube. The effect of a given fluid differs in the case of different organisms, and a fluid chemotactic for one organism may not act on another. Degree of concentration is important, but the nature of the fluid is more so. Of inorganic substances, salts of potassium are the most powerfully attracting. Further, the filtered products of the growth of many bacteria have been found to have powerful chemotactic properties. Corresponding chemotactic pheno-

mena are shown also by certain animal cells, *e.g.* leucocytes, to which reference is made below.

The Parts played by Bacteria in Nature.—As has been said, the chief effect of bacterial action in nature is to break up into more simple combinations the complex molecules of the organic substances which form the bodies of plants and animals, or which are excreted by them. That the very complicated process of putrefaction is due to bacteria was first proved by Pasteur, for any organic substance can be preserved indefinitely from ordinary putrefaction by the adoption of some method of killing all bacteria present in it, as will be afterwards described. This statement, however, does not exclude the fact that molecular changes take place spontaneously in the passing of the organic body from life to death. Many processes not usually referred to as putrefactive are also bacterial in their origin, *e.g.* the souring of milk, the becoming rancid of butter, etc. Bacterial action also underlies many processes of economic importance, such as the ripening of cream and of cheese, and the curing of tobacco.

A certain comparatively small number of bacteria have been proved to be the causal agents in some disease processes occurring in man, animals, and plants. This means that the fluids and tissues of living bodies are, under certain circumstances, a suitable pabulum for the bacteria involved. The effects of the action of these bacteria are analogous to those taking place in the action of the same or other bacteria on dead animal or vegetable matter. The complex organic molecules are modified in constitution or broken up into simpler products. We shall study these processes more in detail later. Meantime we may note that the disease-producing effects of bacteria form the basis of another biological division of the group. Some bacteria are harmless to animals and plants, and apparently under no circumstances give rise to disease in either. These are known as saprophytes. They are normally engaged in breaking up dead animal and vegetable matter. Others normally live on or in the bodies of plants and animals and produce disease. These are known as parasitic bacteria. Sometimes an attempt is made to draw a hard-and-fast line between the *saprophytes* and the *parasites*, and obligatory saprophytes or parasites are spoken of. This is an erroneous distinction. Some bacteria which are normally saprophytes can produce pathogenic effects (*e.g.* B. tetani), and it is consistent with our knowledge that the best-known parasites may have been derived from saprophytes. On the other hand, the fact that most bacteria associated with

disease processes, and proved to be the cause of the latter, can be grown in artificial media, shows that for a time at least such parasites can be saprophytic. As to how far such a saprophytic existence of disease-producing bacteria occurs in nature, we are in many instances still ignorant.

The Methods of Bacterial Action.—The processes which bodies undergo in being split up by bacteria depend, first, on the chemical nature of the bodies involved, and, secondly, on the varieties of the bacteria which are acting. The destruction of albuminous bodies which is mostly involved in the wide and varied process of putrefaction, can be undertaken by different varieties of bacteria. The action of the latter on such substances is analogous to what takes place when albumins are subjected to ordinary gastric and intestinal digestion. In these circumstances, therefore, the production of albumoses, peptones, etc., similar to those of ordinary digestion, can be recognised in putrefying solutions, though the process of destruction always goes further, and simpler substances, *e.g.* creatinin, indol, and, it may be, crystalline bodies of an alkaloidal nature, are the ultimate results. At a later stage still simpler bodies are formed, and there is not infrequently the abundant production of such gases as sulphuretted hydrogen, carbon dioxide, methane, etc. The process is an exceedingly complicated one when it takes place in nature, and different bacteria are concerned in the different stages. Many other bacteria, *e.g.* some pathogenic forms, have a similar digestive capacity, though they may not produce the simpler compounds characteristic of putrefaction. When carbohydrates are being split up, then various alcohols, ethers, and acids (*e.g.* lactic acid) are produced. One common result of bacterial action is thus an alteration of the reaction of a medium, sometimes toward the acid, sometimes toward the alkaline side. Reduction phenomena are also frequently observed. For an exact knowledge of the destructive capacities of any particular bacterium there must be an accurate chemical examination of its effects when it has been grown in artificial media the nature of which is known. Many substances are produced by bacteria, of the exact nature of which we are still ignorant; for example, the *toxins* which play such an important part in the action of many pathogenic species.

Many of the actions of bacteria depend on the production by them of *ferments* of a very varied nature and complicated action. Thus the digestive action on albumins probably depends on the production of a peptic ferment analogous to that produced in the animal stomach. Ferments which invert sugar, which split up

sugars into alcohols or acids, which coagulate casein, which form ammonium carbonate from urea, also occur.

Such ferments may be diffused into the surrounding fluid, or be retained in the cells where they are formed. In the latter case the bacterial protoplasm often must be thoroughly disintegrated, *e.g.* by grinding, before the ferment is liberated. Sometimes the breaking down of the organic matter appears to take place within, or in the immediate proximity of, the bacteria, sometimes wherever the soluble ferments reach the organic substances. And in certain cases the ferments diffusing out into the surrounding medium probably break down the constituents of the latter to some extent, and prepare them for a further, probably intracellular, disintegration. Thus, in certain putrefactions of fibrin, if the process be allowed to go on naturally, the fibrin dissolves and ultimately great gaseous evolution of carbon dioxide and ammonia takes place, but if the bacteria, shortly after the process has begun, are killed or paralysed by chloroform, then only a peptonisation of the fibrin occurs, without the further splitting up and gaseous production. That a purely intracellular digestion may take place is illustrated by what has been shown to occur in the case of the Micrococcus ureæ, which from urea forms ammonium carbonate by adding water to the urea molecule. Here, if after the action has commenced the bacteria are filtered off, no further production of ammonium carbonate takes place, which shows that no ferment has been dissolved out into the urine. If now the bodies of the bacteria be extracted with absolute alcohol or ether, which of course destroy their vitality, a substance is obtained of the nature of a ferment, which, when added to sterile urine, rapidly causes the production of ammonium carbonate. This ferment has evidently been contained within the bacterial cells. According to some, the intracellular ferments alone have the capacity of initiating profound changes in material absorbed, while the easily diffusable agents have only a hydrolysing power. In the investigation of the phenomena of the ferment action of bacteria, it has been noted in certain cases that the ferments formed depend on the food supply offered to the bacterium. Thus in one case a bacterium growing in starch forms diastase, but it does not so do when grown on sugar.

The disintegration of organic material, which is so prominent an effect of bacterial growth, must be a by-effect in the synthesis of the complex substances of which the bacteria themselves are built up. The most striking example of such synthetic power is presented in the case of the bacteria which in the soil make nitrogen more avail-

able for plant nutrition by converting ammonia into nitrites and nitrates. Winogradski, by using media containing non-nitrogenous salts of magnesium, potassium, and ammonium, and free of organic matter, demonstrated the existence of forms which convert, by oxidation, ammonia into nitrites, and of other forms which convert these nitrites into nitrates. Both can derive their necessary carbon from alkaline carbonates. Other bacteria, or organisms allied to the bacteria, exist which can actually take up and combine into new compounds the free nitrogen of the air. These are found in the tubercles which develop on the rootlets of the leguminosæ. Without such organisms the tubercles do not develop, and without the development of the tubercles the plants are poor and stunted. Bacteria thus play an important part in the enrichment and fertilisation of the soil.

Variation and Mutation.—Recent bacteriological literature contains many references to the subject of variation and mutation among the bacteria, and the remarkable diversity of biological types met with in certain groups necessitates careful consideration of this question. A distinction must, of course, be drawn between the "fluctuating" variations in biological characters of different strains round the average or standard characteristic of a particular species, and the development of new types differing in some character from the original and retaining the new feature in successive generations without reversion to the original. To the latter the term "mutant" may justifiably be applied.

Pleomorphism is a common feature of bacterial morphology, and it is well recognised how the microscopic appearances of bacteria may vary somewhat with their environment. Even under the same conditions, individuals tend to differ from one another though presenting an average or standard appearance. Thus a bacillus may vary in length from an almost coccal form to an elongated filament. Variation from the typical morphology is specially marked in culture, and in some cases more so after prolonged artificial culture. Some workers have attached considerable significance to morphological variations and claim to have demonstrated markedly diverse morphological types in single species, and have thus postulated a life-cycle among the bacteria. A filter-passing stage has even been described. It is generally agreed, however, that among the pathogenic bacteria, species are markedly uniform in their standard morphology, and any variations noted have been ascribed to pleomorphism, degeneration forms, and the effects of unfavourable environment. Thus it is generally impossible to select out a strain differing permanently in morphology from the original

organism, though after prolonged artificial culture some departure from the original appearances may be noted.

What may be called physiological variations are of frequent occurrence. In a bacterial growth the individuals are not uniform in character, but vary in their properties, powers of resistance, etc., and when we consider that many generations may occur in twenty-four hours, it is evident how readily variations may come into prominence when the surroundings are partially unfavourable. For example, in the case of the action of the bacteriophage referred to below (p. 28), some of the organisms may withstand lysis and thus a resistant strain may be developed. Some physiological variations are associated with differences in the appearances of the growth. In the coli-typhoid group, attention has been drawn by various observers to the occurrence of variant morphological types of colonies in the same strain, and this variation may be associated with variation in agglutinative properties. The "rough" and "smooth" colony variants described by Arkwright exemplify this type of variation. Corresponding colony variations associated with alterations in virulence have been noted among streptococci (Cowan). Other similar examples might be adduced. Such variations, however, may not be associated with altered appearances. For instance, Andrewes found in the salmonella group that colonies similar in appearance differ in their antigenic structure in relation to agglutination (p. 427). Variations in the biochemical or fermentative actions of bacteria are of common occurrence and have been especially studied in the typhoid-coli group, as will be afterwards described. Thus new fermentative properties may be acquired, and this may occur spontaneously in culture but is met with especially under two conditions, namely, (*a*) when the organisms are grown for a long time on a medium containing a particular constituent, *e.g.* a sugar, or (*b*) when a substance somewhat inimical to growth is added to the medium—in both cases the organisms become adapted to their surroundings. The occurrence of such variations is sometimes indicated by the appearance of papillæ on certain of the colonies, and when an indicator such as neutral red is used along with the fermentable substance, these are of different colour, *e.g.* red papillæ on white colonies. In many of the examples given the variants revert to the original type after subculture, but in certain instances they are permanent—that is, are true mutants. The latter occurrence was observed first by Neisser and by Massini in the case of B. coli mutabilis, and similar phenomena were afterwards observed and fully

described by Penfold, whose papers may be consulted. Twort, by growing the typhoid bacillus for a long time in lactose medium, found that it acquired the property of fermenting this sugar; but this is a rare result and others have failed to effect the change.

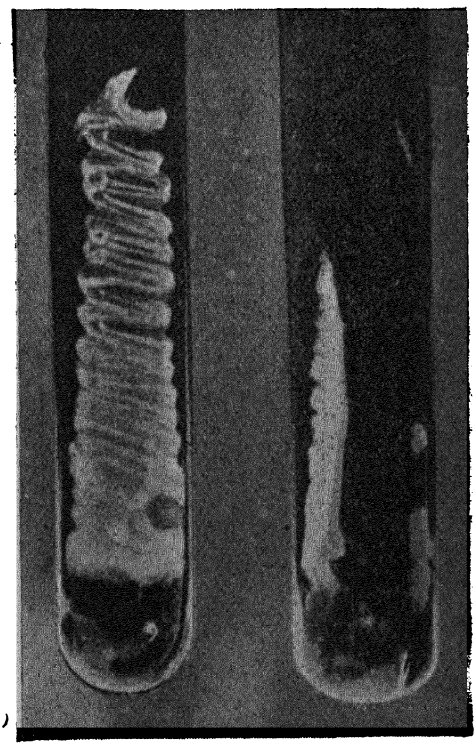
The whole subject of variation and mutation among the bacteria is too extensive for detailed review in this work, but it may be said that, in general, the variations described among the pathogenic bacteria in no way interfere with the accepted biological classification. The species recognised remain true to type; any variations observed represent subsidiary differences from the standard type, and, as it were, only extend the type characters of the various recognised groups.

Bacteriophage

It has long been known that bacteria in culture are apt to undergo a process of autolysis, becoming disintegrated and sometimes disappearing; such changes are much more rapid and pronounced in some species than in others. Within recent years, however, it has come to be recognised that rapid lysis may be set up by certain external agents or stimuli, and that the lytic process is transmissible to other cultures by the products of the lysis. Twort (1915), in working with certain micrococci obtained from vaccine lymph, observed the appearance of glassy and transparent patches in cultures on agar, which increased at the expense of the ordinary bacterial growth. He found that a similar lytic change could be transmitted to fresh growths by inoculation from a glassy patch, and that in this way the change could be continued for an indefinite period of time. Further, he showed that the lytic agent was present in bacterium-free filtrates which had been passed through the finest porcelain filters. He discussed the various possibilities in explanation of the phenomenon, including that of its being due to an ultra-microscopic virus; but considered that a definite conclusion was not warranted. A similar phenomenon was described somewhat later (1917) by d'Herelle, first in the case of a dysentery bacillus, and was made the subject of a long series of important researches. He formed the opinion that the lysis was due to an ultra-microscopic virus and, in fact, represented the result of a parasitic infection of the bacteria by the virus. He applied the term "bacteriophage" to the supposed virus, and this term has come into common use; it is often used in the abbreviated form of "phage."

Method of Obtaining.—Samples of bacteriophage have most

frequently been obtained from the alimentary tract of the human subject and animals, either in a healthy or diseased condition. They have also been got from organisms cultivated from lesions in the tissues, and, further, they have occasionally been found to appear in ordinary cultures in the laboratory. The usual method of obtaining a bacteriophage, say from the intestine, is to make a culture in bouillon, then filter and test for the presence of the lytic property by inoculating a given culture of an organism with the filtrate. After a bacteriophage has been obtained which acts energetically on a given organism, it may be maintained indefinitely, as a rule, by passing from culture to culture. Occasionally, however, it dies out spontaneously. When an agar slope culture is treated with bacteriophage, a clear band develops along the line of inoculation, and this may involve the whole growth, the surface of the medium becoming clear of growth (Fig. 2). Sometimes colonies develop on the clear surface after a time, and these are formed from resistant organisms which have escaped the lytic action. When a bouillon culture, turbid in appearance, is inoculated with bacteriophage, it gradually becomes clear as the lysis goes on and ultimately all trace of turbidity may disappear. If a well-developed young culture of an organism in broth be inoculated, and then some of it be spread over an agar plate the film of growth comes to be beset with small circular clear areas, which represent points of action of the lytic principle or, according to d'Herelle's view, " colonies of the bacteriophage " (Fig. 3). When the bacterial colonies on a plate are of some size, those affected by the lytic process may be partly liquefied and present a " nibbled " appearance. In all these circum-



A *B*

FIG. 2.—*A*. Growth of Staphylococcus albus on agar. *B*. The same after being inoculated with bacteriophage; the dark area is due to lysis of the growth. (Natural size.)

stances, the bacteria affected become granular in appearance, sometimes swelling up considerably, then they undergo a process of lysis and practically disappear. It is to be noted that propagation of the bacteriophage, so far as is known, takes place only in the substance of *living* bacteria; and no one has been able to show any increase of it in any medium not containing the bacteria.

Powers of Resistance.—In comparison with most unicellular organisms, bacteriophage has comparatively high powers of resistance to various agencies. For example, a lysed culture may contain the lytic principle after several years, as is shown by inoculating from it to a fresh culture of the microbe originally lysed. Fæces kept in sealed tubes for over a year have been found to contain the bacteriophage practically unchanged. Further, it can withstand drying for several months at least. Different samples show variation in resistance to heat, but one may say that in some instances a temperature of about 75° C. for half an hour is necessary to ensure destruction. It shows also considerable resistance to treatment with certain agents such as acetone, alcohol, ether, etc. For instance, d'Herelle found that it was not destroyed after being kept in 1 : 200 corrosive sublimate or in 1 : 100 carbolic acid for three days; after a somewhat longer period destruction followed. Neutral salts of quinine are active in destroying the activity, 3 per cent. solutions being effective in thirty minutes. Kabéshima showed that when a lysed culture was precipitated by acetone, a fine powder was thrown down and that this contained the lytic agent unchanged for about six weeks. It may also be mentioned that bacteriophage persists for a long period in strong

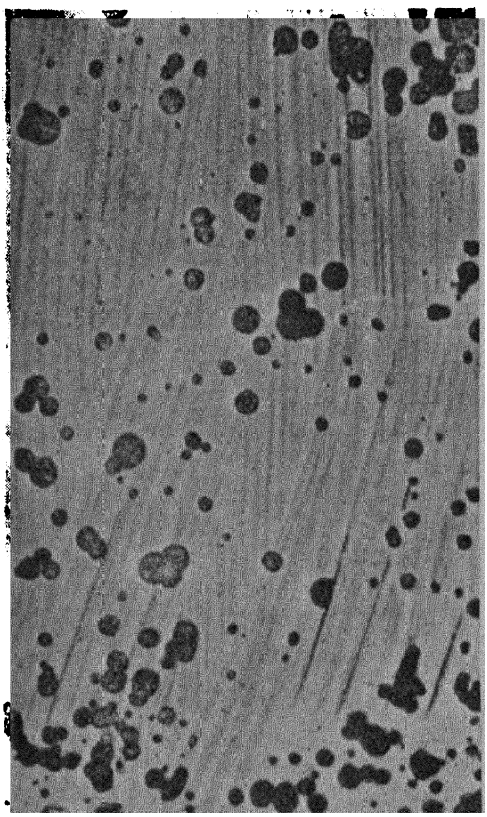


FIG. 3.—Plate culture of staphylococcus with "colonies" of bacteriophage; the latter appear as dark spots on the pale background of growth.

solutions of glycerine, in this respect corresponding to the filterable viruses.

Nature.—Regarding the true nature of the transmissible lytic agent there has been, and still is, much controversy, and at present it does not seem justifiable to express a definite view. Various theories have been put forward, but these essentially centre around two, namely, (*a*) that the agent is a living ultramicroscopic virus, and (*b*) that it is a non-living ferment supplied by the bacteria themselves. All are agreed that the actual lytic process depends upon an enzyme; the question at issue is whether it is produced by a definite virus, or whether it is formed by the bacteria themselves. With regard to the former, one has to note that the supposed virus is beyond the limits of microscopic vision, and as yet it has not been observed directly, still less its multiplication by division. Furthermore, such a virus has never been cultivated in any medium apart from the susceptible bacteria in the living state; and lastly, the facts with regard to resistance to various agents do not appear to correspond with those of any known living organism. In these circumstances it does not seem justifiable to assume a living organism unless there is no possibility of other explanation. Those who regard the agent as non-living consider that it is an autolytic enzyme transmissible from bacterium to bacterium. According to Bordet and Ciuca, who obtained a bacteriophage in a peritoneal exudate in the guinea-pig produced by B. coli, some external agent, probably leucocytic in origin, modifies the bacteria so that an autolytic process is started. This modification is transmitted to the descendants of the bacteria, and thus the lytic process is described as being hereditarily transmissible. The autolysin diffuses in the medium and sets up similar autolysis in other bacteria—the process is thus in a sense contagious. The view of Kabéshima is closely similar. He supposes that the autolysin exists in the bacteria as a pro-diastase and that this may be activated by a ferment, probably supplied by a digestive gland in connection with the alimentary canal. The activated lysin then continues a similar process in other bacteria. Such views involve the possibility of an enzyme giving rise to, or setting free, more enzyme of similar nature, as the lytic agent undoubtedly increases in amount.

Bacteriophages obtained from different sources present considerable differences in respect to the organisms on which they exert a lytic action. A phage active against the bacillus of dysentery, for instance, has, as a rule, a restricted action on

members of the same group, especially on varieties of B. coli ; as a rule it has little or no action on the organisms of other groups. It has been found, however, that when a phage is added repeatedly to cultures of a resistant organism lytic properties towards the latter may be developed. D'Herelle showed, for example, that a phage for the dysentery bacillus may in this way become active for a staphylococcus. Further, it has been found that when lytic action for one organism is acquired it may be lost for another. He regards the change in the lytic properties as a process of adaptation and as evidence that the phage is a living organism. The phenomenon seems, however, explicable also on the enzyme theory, since if one enzyme can lead to the activation of another, a more active enzyme may be set free and its effects will become increasingly manifest. In any case, a phenomenon allied to adaptation is an observed fact. It seems fruitless at present to discuss whether or not there are several phages—the question as to unity or plurality. D'Herelle holds that there is one phage of which there are several varieties. In support of this view he has found that an antiserum to the phage of the dysentery bacillus gives a complement-fixation reaction (p. 134) with phages active to other organisms. Such a result is supposed by him to indicate an essential similarity in the phages. The question, however, is beset with many difficulties, and it is sufficient at present to recognise that phages from different sources vary greatly in their lytic effects, and that these may become modified by conditions of growth.

CHAPTER II

METHODS OF CULTIVATION OF BACTERIA

Introductory.—To obtain pure cultures is the first requisite of bacteriological research. Now, as bacteria are practically omnipresent, we must first of all have means of destroying all extraneous organisms which may be present in the food media to be used, in the vessels in which these media are contained, and on all instruments which are to come in contact with our cultures. The technique of this destructive process is called sterilisation. We must therefore study the *methods of sterilisation.* The growth of bacteria in other than their natural surroundings involves further the *preparation of sterile artificial food media,* and when we have such media prepared we have still to consider the technique of the *separation of micro-organisms from mixtures of these, and the maintaining of pure cultures when the latter have been obtained.* We shall find that different methods are necessary according as we are dealing with *aerobes* or *anaerobes.* Each of these methods will be considered in turn.

Methods of Sterilisation

To exclude extraneous organisms, all food materials, glass vessels containing them, wires used in transferring bacteria from one culture medium to another, instruments used in making autopsies, etc., must be sterilised. These objects being so different, various methods are necessary, but underlying these methods is the general principle that all bacteria are destroyed by heat. The temperature necessary varies with different bacteria, and the vehicle of heat is also of great importance. The two vehicles employed are hot air and hot water or steam. The former is usually referred to as "dry heat," the latter as "moist heat." As showing the different effects of the two vehicles, Koch found, for instance, that the spores of Bacillus anthracis, which were killed by moist heat at 100° C., within one hour, required three hours' dry heat at 140° C. to effect death. Both forms of heat may be applied at different temperatures—

in the case of moist heat above 100° C., a pressure higher than that of the atmosphere must of course be developed.

A. *Sterilisation by Dry Heat*

A (1). Red Heat and Flaming.—Red heat is used for the sterilisation of platinum needles. A dull red heat is used for cauteries, or the points of forceps. Similarly, small objects may be sterilised by "flaming," *i.e.* passing them repeatedly through the flame; this method is used for cover-slips, slides, the mouth of culture tubes, cotton-wool stoppers, paper, etc.; but care must be taken not to melt or char the objects. Needles and scalpels may be sterilised by dipping them in alcohol, which is then burned off.

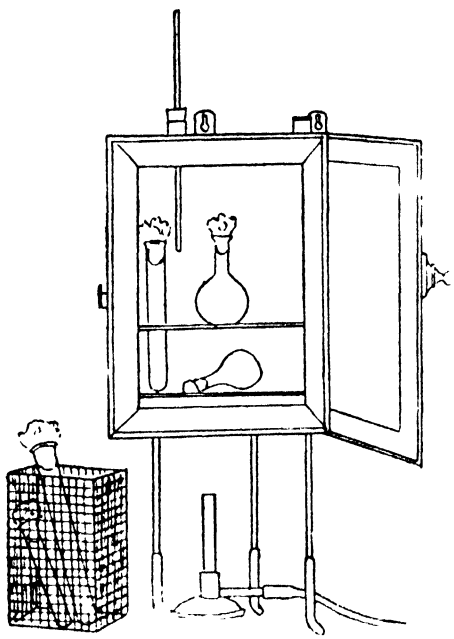


FIG. 4.—Hot-air steriliser (simple form).

A (2). Sterilisation by Dry Heat in a Hot-Air Chamber.—The chamber (Fig. 4) consists of an outer and inner case of sheet iron. In the bottom of the outer there is a large hole. A Bunsen is lit beneath this, and thus plays on the bottom of the inner case, round all the sides of which the hot air rises and escapes through holes in the top of the outer case. A thermometer passes down into the interior of the chamber, half-way up which its bulb should be situated. It is found, as a matter of experience, that an exposure in such a chamber for one hour to a temperature of 160° C. is sufficient to kill all the organisms which usually pollute articles in a bacteriological laboratory, though circumstances might arise where this would be insufficient. Electrically heated ovens are now often used; by means of a regulator the temperature can be maintained at the desired height. This means of sterilisation is used for glass apparatus, *e.g.* flasks, test-tubes, Petri dishes, pipettes, and throat swabs. Cotton-wool plugs are not damaged by the temperatures employed and should be inserted before heating. Such pieces of apparatus are thus obtained sterile and dry. It is

advisable to have the glass vessels dry and to put them into the chamber before heating it, and to allow them to stand in it after sterilisation till the temperature falls. Sudden heating or cooling is apt to cause glass to crack. The method is manifestly unsuitable for culture media which contain water.

B. *Sterilisation by Moist Heat*

B (1). **By Boiling.**—The boiling of a liquid for five minutes is sufficient to kill all organisms if no spores be present, but some spores may resist boiling for an hour and a half. This method is useful for sterilising distilled or tap water, and for tubes, syringes, instruments, etc. To minimise rusting of knives and steel instruments it is well to boil the water for some time before placing them in it. The form of steriliser commonly used is an enamel-ware fish-kettle pattern, which should be provided with a removable tray with a raised edge, to prevent articles from falling off.

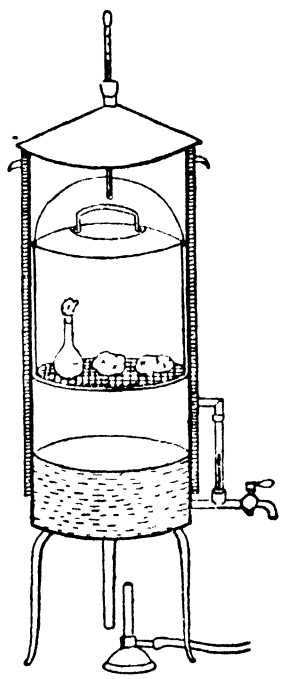


FIG. 5.—Koch's steam steriliser (simple form) in section.

B (2). **By Steam at 100° C.**—This is by far the most useful means of sterilisation for culture media. The apparatus ordinarily used is " Koch's steam steriliser " (Fig. 5). This in its simplest form consists of a tall metal cylinder on legs, provided with a lid, and covered externally by some bad conductor of heat, such as felt or asbestos. A perforated tin diaphragm is fitted in the interior at a little distance above the bottom, and there is a tap at the bottom by which water may be supplied or withdrawn. If water to the depth of 3 inches be placed in the interior and heat applied, it will quickly boil, and the steam streaming up will surround any flask or other object standing on the diaphragm. Here no evaporation takes place from any medium, as it is surrounded during sterilisation by an atmosphere saturated with water vapour. It is convenient to have the cylinder tall enough to hold a litre flask with a funnel 7 inches in diameter standing in its neck. The funnel may be supported by passing its tube through a second perforated diaphragm placed in the upper part of the steam chamber.

With such a " Koch " in the laboratory a hot-water filter is not needed. The Koch's steriliser may be heated by passing into the water steam from a steam-heating system or by electricity. If the Koch is attached to the cold water supply pipe by a ball-cock the water in it will be maintained automatically at a constant level. One and a half hour's steaming will usually sterilise any watery fluid, but in the case of media containing gelatin such an exposure is not practicable, as, with long boiling, gelatin tends to lose its physical property of solidification. The method adopted in this case is to *steam for twenty minutes on each of three succeeding days.*

This is a modification of what is known as " Tyndall's intermittent sterilisation." The fundamental principle of this method is that all bacteria in a non-spored form are killed by the temperature of boiling water, while if in a spored form they may not be thus killed. Thus by the sterilisation on the first day all the non-spored forms are destroyed —the spores remaining alive. During the twenty-four hours which intervene before the next heating, these spores, being in a favourable medium, are likely to assume the non-spored form. The next heating kills these. In case any may still not have changed their spored form, the process is repeated on a third day. Experience shows that usually the medium can now be kept indefinitely in a sterile condition.

Steam at 100° C. is therefore available for the sterilisation of all ordinary media. In using the Koch's steriliser, especially when a large bulk is to be sterilised, it is best to put the medium in while the apparatus is cold, in order to make certain that the whole of the mass reaches the temperature of 100° C. The period of exposure is reckoned from the time boiling commences in the water in the steriliser. At any rate, allowance must always be made for the time required to raise the temperature of the medium to that of the steam surrounding it. It is advisable to cover cotton-wool stoppers of flasks and tubes with several layers of parchment or " Kraft " paper to prevent excessive wetting.

B (3). Sterilisation by Steam at High Pressure.—This is the most rapid and effective means of sterilisation. It is effected in an autoclave (Fig. 6). This is a gun-metal cylinder supported in a cylindrical sheet-iron case ; its top is fastened down with screws and nuts and rendered airtight by an asbestos washer ; it is furnished with a safety-valve, tap, and pressure-gauge. As in Koch's steriliser, the contents are supported on a perforated diaphragm. The source of heat is a large Bunsen beneath, or the autoclave may be heated by electricity or from a steam heating system. The temperature employed is usually 115° C.

or 120° C. To boil at 115° C., water requires a pressure of about 23 lb. to the square inch (*i.e.* 8 lb. plus the 15 lb. of ordinary atmospheric pressure). To boil at 120° C., a pressure of about 30 lb. (*i.e.* 15 lb. plus the usual pressure) is necessary. In such an apparatus the desired temperature is maintained by adjusting the safety-valve so as to blow off at the corresponding pressure. One exposure of media to such temperatures for a quarter of an hour is sufficient to kill all organisms or spores. Here, again, care must be taken when gelatin is to be sterilised, or when the medium contains substances which are readily decomposed, *e.g.* carbohydrates. These must not be exposed to temperatures above 105° C., and are best sterilised by the intermittent method. Certain precautions are necessary in using the autoclave. Care must be taken to ensure the presence of sufficient water, so that there is a residuum when steam is fully up, otherwise the steam is superheated, and the pressure on the gauge does not indicate the temperature correctly. The procedure in using the autoclave is as follows: Insert the materials to be sterilised; then fix down the lid and apply the heat. Leave the tap open until a steady jet of steam escapes from it, since if care is not taken to expel all the air initially present, a mixture of air and steam being present, the pressure read off the gauge cannot be accepted as an accurate indication of the temperature. Then turn off the tap and reckon the time of sterilisation from this point. Finally, after ceasing to apply the heat, in all cases it is necessary to allow the apparatus to cool well below 100° C. before opening it or allowing steam to blow off, otherwise there will be a sudden development of steam when the pressure is removed, and fluid media will be blown out of the flasks. In order to prevent cotton-wool stoppers from becoming wet they should be covered with several layers of parchment or "Kraft" paper. Autoclaving is the best method of rendering innocuous infected material, such as old cultures, especially when spores are present.

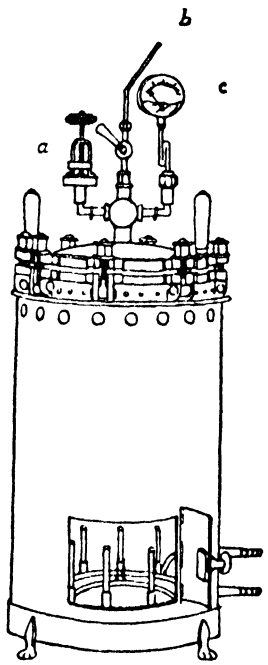


FIG. 6.—Autoclave,
a. Safety-valve.
b. Tap
c. Gauge.

B (4). **Sterilisation at Low Temperatures.**—Many organisms in a non-spored form are killed by a prolonged exposure to a temperature of 57° C. This fact has been taken advantage of

for the sterilisation of blood serum or other materials containing protein, which will coagulate if exposed to a temperature above that point. Such a medium is sterilised on Tyndall's principle by exposing it for an hour at 57° C., for eight consecutive days, it being allowed to cool in the interval to the room temperature. This is best effected in a water-bath heated by means of gas or electricity and provided with a temperature regulator.

Serum sterilisers are constructed in which the test-tubes are placed in the sloped position, and in which inspissation (*vide* p. 52) can afterwards be performed at a higher temperature. Bacterial cultures which are intended for use as vaccines are sterilised by heating in a water-bath, the temperature of which should be as low as is compatible with killing the organisms, *e.g.* for one hour at 60° C. in most cases. Overheating should be avoided, as the immunising power tends to be damaged thereby.

C. Sterilisation by Chemicals.—Volatile antiseptics such as chloroform may be used to preserve serum which is to be kept for preparing media : 5 c.c. chloroform are added to each 100 c.c. of serum, and the mixture, in a stoppered bottle, is shaken well at frequent intervals for several days and is then stored at room temperature. Before use all undissolved chloroform is removed from the serum ; the latter may then be exposed in a thin layer at 57° C., but this is not essential when Löffler's medium is to be made, as the remaining chloroform volatilises in the process of slow heating required for coagulating and sterilising the serum.

D. Sterilisation by Filtration.—In some cases fluids, such as serum, are sterilised most effectively by filtration through filters whose pores are too small to let bacteria pass. Three types of filter are in common use, namely, those of unglazed porcelain (Chamberland type), those of diatomaceous earth (*e.g.* the Berkefeld filter), and those composed of fibrous material (the Seitz filter). The last type is the cheapest and most convenient for sterilising considerable quantities of fluid. It is essential always to test the filtered material for sterility by incubating it for several days at 37° C. with or without the addition of culture medium. Filtration is dealt with later (p. 86).

Maintenance of Sterility

It is essential to prevent recontamination of sterilised materials, *e.g.* from the dust in the air. For this reason, flasks, test-tubes, etc., are closed with firmly fitting stoppers of cotton-wool which are inserted before sterilising. When the contents have to be

kept sterile for a considerable time, it is well also to cover the stoppers with sterile paper which is tied on round the neck of the vessel, or with indiarubber caps. Petri dishes and pipettes should be wrapped in paper before sterilising, and if they are to be stored they should be sterilised in metal boxes with close-fitting lids. Many articles, such as capillary pipettes, are conveniently sterilised and stored in wide glass "boiling" tubes plugged with cotton wool.

The Preparation of Ordinary Culture Media

The general principle to be observed in the artificial culture of bacteria is that the medium used should resemble that on which the bacterium grows naturally. In the case of pathogenic bacteria the medium therefore should resemble the juices of the body. Thus blood serum is often used. Other media have been found which can support the life of most of the pathogenic bacteria. These consist of proteins or carbohydrates in a fluid, semi-solid, or solid form. It is an advantage to have a variety of media, since growth characters on particular media, non-growth on some and growth on others, etc., constitute specific differences which are valuable in the identification of bacteria. The most commonly used media have as their basis a watery extract of meat. Most bacteria in growing in such an extract cause only a grey turbidity. A great advance resulted when Koch, by adding to it gelatin, provided a transparent solid medium in which growth characteristics of particular bacteria become evident. Many organisms, however, grow best at a temperature at which this nutrient gelatin is fluid, and therefore another gelatinous substance called agar, which does not melt below 98° C., was substituted. Bouillon made from meat extract, gelatin, and agar media, and the modifications of these, constitute the chief materials in which bacteria are grown.

Preparation of Meat Extract

The flesh of the ox, calf, or horse is usually employed. Horse-flesh has the advantage of being cheaper and containing less fat than the others; though generally quite suitable, it has the disadvantage for certain purposes of containing a larger proportion of fermentable sugar. The flesh must be freed from fat, and finely minced. To a pound of mince add 1000 c.c. distilled water, and mix thoroughly in a shallow dish. Set aside in a cool place for twenty-four hours. Skim off any fat present, removing the last traces by stroking the surface of the fluid with pieces of filter paper. Place a clean linen cloth over the mouth of a large filter funnel, and strain the fluid through it into a flask. Pour the minced meat into

the cloth, and, gathering up the edges of the latter in the left hand, squeeze out the juice still held back in the contained meat. Finish this expression by putting the cloth and its contents into a meat press, similar to that used by pharmacists in preparing extracts; thus squeeze out the last drops. The resulting fluid contains the soluble albumins of the meat, the soluble salts, extractives, and colouring matter, chiefly hæmoglobin. It is now boiled thoroughly for three hours, by which process the albumins coagulable by heat are coagulated. Allow it to cool, and filter through Chardin's filter paper.[1] Make up to 1000 c.c. with distilled water. The resulting fluid ought to be quite transparent, of a yellowish colour without any red tint. If there is any redness, the fluid must be reboiled and filtered till this colour disappears, otherwise in the later stages it will become opalescent. A large quantity of the extract may be made at a time, and what is not immediately required is put into a large flask, the neck plugged with cotton wool, and the whole sterilised by methods B (2) or (3). This extract contains very little albuminous matter, and consists chiefly of the soluble salts of the muscle, certain extractives, and altered colouring matters, along with any traces of soluble protein not coagulated by heat. It is of acid reaction. The commercial meat extract "Lab-Lemco" added to water in the proportion of 10 grams to the litre forms a very satisfactory substitute for the meat extract described above. By the addition to the meat extract of protein (in the form of a commercial preparation of peptone) and other matter, proper culture media may be obtained. But it has been found that by partially digesting minced meat or casein with trypsin a very suitable substitute for meat extract and peptone results (p. 41).

1. **Bouillon Media.**—These consist of meat extract with the addition of certain substances to render them suitable for the growth of bacteria.

1 (*a*). **Peptone Broth or Bouillon.**—This has the composition—

Meat extract	1000 c.c.
Sodium chloride	5 grams.
Peptone [2]	10 ,,

Boil till the ingredients are dissolved, and make slightly alkaline to litmus as directed below. After alkalinisation, filter through filter paper [3] into flasks, make up to original volume with distilled water, plug the flasks with cotton wool, and sterilise by methods B (2) or (3) (pp. 35, 36)

[1] The original French *Papier Chardin* in unfolded sheets should be used.

[2] For the preparation of ordinary broth or agar medium commercial peptone, *e.g.* Fairchild's, may be used. But where the medium is to be used for testing the fermentation of sugars or the production of indol by organisms it is essential to test the peptone for the absence of these substances; peptones which have been found free from these impurities are Fairchild's peptone "for bacteriological purposes," Chapoteaut's, and Savory & Moore's.

[3] Balston's "Whatman No. 1," filter paper is suitable.

In this medium the place of the original albumins of the meat is taken by peptone, a soluble protein not coagulated by heat. Here it may be remarked that the commercial peptone is not pure peptone, but a mixture of albumoses with a variable amount of pure peptone. The addition of the sodium chloride is necessitated by the fact that alkalinisation precipitates some of the phosphates and carbonates present. Experience has shown that, as a rule, sodium chloride can quite well be substituted. The reason for the alkalinisation is that it is found that most bacteria grow best on a medium slightly alkaline to litmus. Some, *e.g.* the cholera vibrio, meningococcus, are very sensitive to the reaction of their surroundings.

Digest Bases for Media

Horse Heart Digest (Short Method of Preparation—modified from Douglas).—Horse heart (freed from fat and fibrous tissue) is minced finely and is mixed thoroughly with water in the proportion of 1 lb. mince to 1 litre water. The mixture is rendered strongly alkaline to litmus paper with 4 per cent. NaOH solution, and is allowed to stand at room temperature for half an hour, when the reaction is again tested, more alkali being added should the mixture be acid; it is now heated in the Koch's steriliser at 80° C. for half an hour. Then it is cooled to 40° C. and 5 c.c. "liquor trypsinæ co."[1] and 10 c.c. chloroform are added; the mixture is well shaken several times and is allowed to stand at room temperature for fifteen minutes before being placed in the incubator. After incubating at 37° C. for twenty-four hours sufficient 10 per cent. HCl is added to render the reaction acid to litmus paper. The mixture is placed in a flask, which should be about two-thirds full, in the cold Koch and the burner is lighted, so that the temperature is raised slowly and the chloroform evaporates completely; finally it is boiled in the Koch for one and a half hours and thereafter is cooled and filtered. The filtrate is made up to 1 litre with distilled water and 6 grams NaCl are added. This solution constitutes horse heart digest. It may be sterilised at 115° C. for fifteen minutes in plugged flasks and stored for the preparation of broth, agar, etc.

Heart Digest Broth is prepared by adding to the above heart digest 0·5 per cent. NaCl and sufficient 4 per cent. caustic soda solution to render the reaction distinctly alkaline to litmus paper.

Long Digest is prepared as above, but the mixture with trypsin is kept in a loosely stoppered bottle at 37° C. for twenty-one days; after the first ten days' incubation a further quantity of 5 c.c. liquor trypsinæ co. is added. From the commencement the reaction must be tested daily with litmus paper and 4 per cent. NaOH solution added as required to keep the mixture alkaline, since an acid reaction destroys the trypsin and so prevents progress of the digestion.

Casein Digest.—Mix 200 grams "laitproto No. 6 for bacterio-

[1] This preparation of trypsin is made by Allen & Hanburys, 7 Vere Street, Cavendish Square, London, W. Hartley found that owing to its containing glycerol, it is unsuitable for media for certain purposes, *e.g.* for obtaining diphtheria toxin, when trypsin prepared from pig's pancreas should be used, as recommended by Cole and Onslow (p. 50).

logical purposes"[1] into a smooth paste and make up to 1000 c.c. with cold water; then add slowly a boiling solution of 20 grams anhydrous sodium carbonate in 500 c.c. water, stirring vigorously: pour the mixture into a flask and heat in the Koch at 100° C. for ten minutes. Transfer the mixture to a large bottle and wash out the flask with 500 c.c. water, which is also transferred to the bottle. Cool to 40° C., add 50 c.c. liquor trypsinæ co. and 15 c.c. chloroform; shake well and keep the bottle loosely stoppered at 37° C. for five days (shaking daily): then add further 50 c.c. liquor trypsinæ co. and keep the mixture at 37° C. for ten days further. Finally shake well to break up tyrosine masses and pour the whole into a large flask; add 400 c.c. N/1 HCl, steam for thirty minutes and filter; then add to the filtrate 120 c.c. N/1 NaOH and finally adjust the reaction to slight alkalinity as tested with litmus paper, and sterilise.

Casein Digest Broth is prepared by adding 1000 c.c. of the above digest to 2000 c.c. 0·5 per cent. sodium chloride solution, adjusting the reaction and sterilising.

Media prepared with digests as a basis yield much more luxuriant growths of most organisms than do those prepared from ordinary meat extract with peptone; but the latter are preferable for keeping stock cultures, as they maintain the viability of the bacteria for a longer time.

Adjustment of the Reaction of Media.—The adjustment of the reaction of bacteriological media is a matter of great complexity. The method usually adopted with meat extract, which as prepared above is ordinarily slightly acid, is to add saturated sodium carbonate or sodium hydrate solution till the medium is slightly but distinctly alkaline to red litmus paper and no longer affects blue litmus paper. The occurrence of an amphoteric reaction—*i.e.* one where red litmus is turned blue, and blue, red—is thus avoided. The test paper must be immersed in the liquid—on no account is the sampling to be done by transferring drops to the paper by means of a glass rod. The disadvantages of this method are that ordinary litmus is not a delicate indicator and, further, no standardisation of the proper tint to be aimed at is possible. The latter difficulty can be sufficiently got over by making up a solution of disodic phosphate ($Na_2HPO_4.2H_2O$), 11·876 grams to the litre; test paper immersed in this assumes a tint just on the alkaline side of what is usually regarded as the optimum reaction for bacterial growth. In applying this method it is preferable to use azolitmin papers (made by immersing filter paper in 0·1 per cent. of the dye overnight and drying) or neutral-red papers (made by treating the paper with 0·02 per cent. neutral red for three minutes). Both of these papers are more sensitive than ordinary litmus paper.

[1] Obtainable from Casein Ltd., Culvert Works, Battersea, London, S.W.

Eyre's Method.—Several methods have been introduced for adjusting the reaction by titration, and that of Eyre is widely used. It is applicable to any of the media ordinarily employed. The reaction of a medium may be conveniently expressed by the sign + or − to indicate acid or alkaline respectively, and a number to indicate the number of cubic centimetres of normal alkaline or acid solution necessary to make a litre of the medium neutral to phenol-phthalein. Thus, for example, "reaction = −15," will mean that the medium is alkaline, and requires 15 c.c. of normal HCl to make a litre neutral. It has been found that when a medium such as bouillon reacts neutral to litmus, its reaction to phenol-phthalein, according to the above standard, is on the average +25. Now, as litmus was originally introduced by Koch, and as nearly all bacterial research has been done with media tested by litmus, it is evidently difficult to say exactly what precise degree of alkalinity is the optimum for bacterial growth. It is probable that when a medium has been rendered neutral to phenol-phthalein by the addition of NaOH, the optimum degree is generally attained by the addition of from 10 to 15 c.c. of normal HCl per litre, *i.e.* the optimum reaction is from +10 to +15. According to Fuller, the optimum reaction for bacterial growth lies about midway between the neutral point indicated by phenol-phthalein and the neutral point indicated by litmus.

Method.—The medium with all its constituents dissolved is filtered and then heated for about forty-five minutes in the steamer, the maximum acidity being reached after this time. Of the warm medium take 25 c.c. and put in a porcelain dish, add 25 c.c. distilled water, and 1 c c. phenol-phthalein solution. Run in decinormal soda till neutral point is reached, indicated by the first trace of pink colour, the mixture being kept hot.[1] Repeat process thrice, and take the mean; this divided by 10 will give the amount (x) of *normal* soda required to neutralise 25 c.c. of medium; then $40x$ = amount necessary to neutralise a litre; and $40x - 10$ = amount of normal soda necessary to give a litre its optimum reaction. Then measure the amount of medium to be dealt with, and add the requisite amount of soda solution.

Eyre uses a soda solution of ten times normal strength, which is delivered out of a 1 c.c. pipette divided into hundredths; this obviates, to a large extent, the error introduced by increasing the bulk of the medium if a weaker neutralising solution be used. In

[1] The beginner may find considerable difficulty in recognising the first tint of pink in the yellow bouillon. A good way of getting over this is to take two samples of the medium, adding the indicator to one only; then to run the soda into these from separate burettes; for each few drops run into the medium containing the indicator the same amount is run into the other. Thus the recognition of the first permanent change in tint will be at once recognised by comparing the two samples.

using these strong solutions care must be taken to remove any fluid adhering to the *outside* of the pipette. When the acid or alkali has been added the reaction of the medium must be again taken before sterilisation.

Estimation of True Acidity or Hydrogen-Ion Concentration.—The true acidity of any fluid depends on the number of free or dissociated hydrogen ions which it contains, or, as it is ordinarily expressed, on the *hydrogen-ion concentration.* The greater the number of free hydrogen ions the greater is the acidity; the stronger an acid is, the larger is the proportion of its hydrogen in the free or ionised state. The standard solution (in relation to which the concentration is expressed) is 1 gram of free hydrogen ions in a litre of water. Such a standard would correspond with a normal solution of strong acid, say hydrochloric acid (36·5 grams HCl in a litre of water, H = 1 gram), provided that the hydrogen was completely dissociated. As a matter of fact, however, not all the hydrogen is in the form of free ions. In a similar way the hydrogen-ion concentration of decinormal hydrochloric acid is slightly less than a tenth of the standard, but when the solution of acid is very dilute nearly all the hydrogen ions are free. In the case of a weak acid, however, the hydrogen-ion concentration is often only a fraction of that of hydrochloric acid. Accordingly, no definite relationship exists between normal, decinormal, etc. solutions of acids and their true acidity.

True alkalinity, in a corresponding fashion, depends on hydroxyl-ion concentration, and a fluid is neutral when it contains an equal number of free hydrogen and free hydroxyl ions. Pure distilled water is, of course, such a neutral fluid; only a very small proportion of its hydrogen is in the ionised state, the hydrogen-ion concentration being $\frac{1}{10^7}$, that is, 10^{-7}, and its hydroxyl-ion concentration is, of course, the same. According to the system now ordinarily used, the hydrogen-ion concentration, represented as P_H, is expressed by the logarithm of the concentration with its sign changed. Thus, in the case of a neutral fluid, *e.g.* distilled water, the hydrogen-ion concentration is 10^{-7}; its logarithm is -7, and therefore its $P_H = 7$. Similarly, if we used an exponent for the hydroxyl-ion concentration, the P_{OH} of a neutral fluid would be 7 also. Further, the product of hydrogen-ion concentration and hydroxyl-ion concentration is a constant, and $P_H + P_{OH}$ always $= 14$. The reaction of a fluid is ordinarily expressed as P_H, but the true alkalinity (P_{OH}) can always be obtained as it $= 14 - P_H$. Owing to the conventional

change in the sign, the P_H increases as the acidity diminishes, and any P_H greater than 7 means an alkaline reaction ; that is, the fluid contains more hydroxyl ions than hydrogen ions. For example, the blood serum and the tissue fluids, which are slightly alkaline, have a P_H of approximately 7·5 ; and, as a rule, this is the optimum reaction of media for the growth of pathogenic bacteria.

It is necessary that the student should understand that there is an important difference between the acidity of a solution as estimated by the amount of standard solution of alkali necessary to neutralise it and the true acidity, *i.e.* between the " titration acidity " and the hydrogen-ion concentration. For example, $\frac{N}{10}$ acetic acid requires as much alkali to neutralise it as $\frac{N}{10}$ hydrochloric acid, when tested in the usual way by an indicator ; whereas the hydrochloric acid contains several times the number of free hydrogen ions which acetic acid does—that is, is several times as strong an acid.

Another important subject is that of *buffers* in solution, that is, substances which diminish the amount of free ions when they are added to a solution. When, for instance, a certain amount of hydrochloric acid is added to a solution of sodium acetate, sodium chloride is formed with the setting free of acetic acid and water, and the acetic acid is less ionised than the hydrochloric acid ; thus the mixture contains fewer free hydrogen ions than the acid added. Amongst such substances which act as buffers are various salts, like carbonates, phosphates, citrates, etc., amino-acids and proteins ; all these act in the way indicated. Buffer substances are abundantly present in ordinary bacterial media, and thus, even when the true neutral point is known, the desired P_H cannot be obtained merely by the proportional addition of a standard solution of acid or alkali.

Estimation of P_H.—The P_H of a solution can be directly determined only by means of an electrical method, involving the use of the hydrogen electrometer. Such a method is impracticable for ordinary bacteriological laboratories, and a colorimetric method which gives satisfactory results is ordinarily used instead. This depends on the fact that when indicators are changed in colour by the reaction of a medium, there are two points in P_H between which the change takes place and that a particular tint given by an indicator corresponds with a definite P_H. The procedure is thus to determine what amount of alkali is neces-

sary to bring a given amount of the medium to a tint corresponding with the desired P_H; alkali is then added to the whole volume of the medium in proportionate amount.

Solutions of graded P_H can be suitably prepared by mixing $\frac{M}{15}$ di-sodium phosphate (Na_2HPO_4) and $\frac{M}{15}$ mono-potassium phosphate (KH_2PO_4) in varying proportions. Such solutions with a suitable indicator added, say phenol red, can be purchased. They are supplied in hard white glass tubes—the so-called "cordite" tubes—of uniform diameter and thickness of glass. And similar tubes are used in making comparative

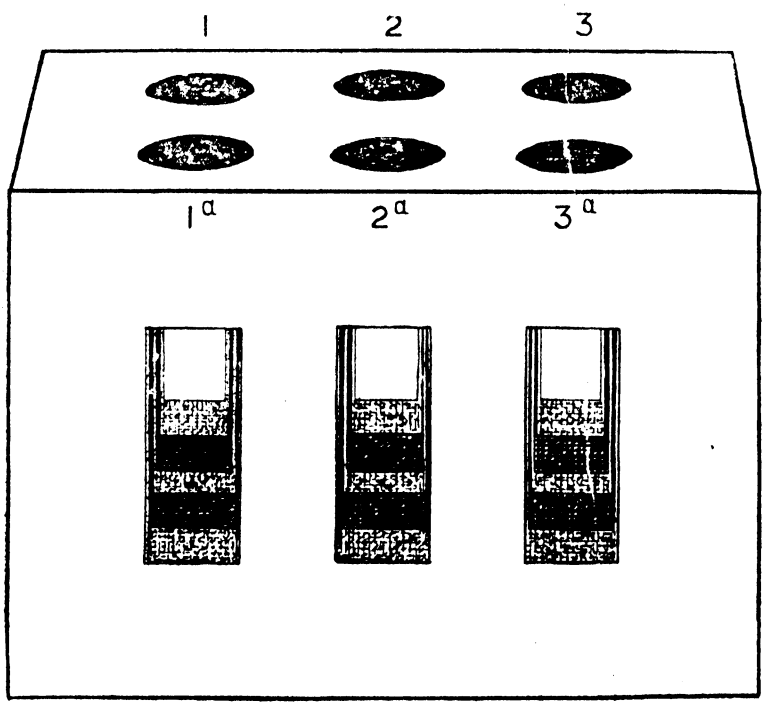


FIG. 7.—Comparator used in estimation of hydrogen-ion concentration.

estimations. For example, a series of tubes with P_H 6·8—8·4 (with differences of 0·2) with phenol red show variations in tint from pale yellow to crimson. We have, in the first place, to ascertain how much alkali has to be added to a given quantity of medium—5 c.c., in order to bring it to the colour of the desired reaction, say $P_H = 7·5$. In such a case it is convenient to use two tubes with $P_H = 7·4$ and $= 7·6$ respectively, and to bring the tint of the medium to a point intermediate between the two. A comparator rack to hold two rows of tubes is used and is so arranged that each pair of tubes (front and back row) can be superimposed and examined by light transmitted through them.

The scheme of such a comparator is shown in Fig. 7. In 1 and 3, two standard tubes with $P_H = 7{\cdot}4$ and $= 7{\cdot}6$ are placed; each contains phenol red to show the corresponding tint. In front of each in 1*a* and 3*a* a tube of medium is placed; in 2 a tube of distilled water, and in 2*a* a tube containing 5 c.c. of medium and 0·5 c.c. of a 0·01 per cent. solution of phenol red. It is thus evident that the light transmitted through each pair of tubes passes through the same constituents, namely, (*a*) a tube of medium, (*b*) a tube of water or colourless solution, and (*c*) a solution of phenol red; the difference being that in the case of the standard tubes the indicator is in the phosphate solution, whereas in the tubes to be tested the indicator is in the medium. The alkali to be added is in the form of $\frac{N}{20}$ sodium hydrate solution, with phenol red added. This is prepared by taking 500 c.c. of $\frac{N}{10}$ NaOH, adding 91 c.c. of 0·01 per cent. phenol red, and making up to 1000 c.c. with distilled water. A microburette is necessary to deliver and measure the solution of alkali. For this purpose a 1 c.c. pipette graduated to 0·1 c.c. may be used; a fine pipette is attached to the lower end by a piece of rubber tubing with a pinchcock on the rubber to control delivery.

Method.—Before standardising, it is convenient to bring the reaction of the medium to about $P_H = 7$ by the addition of sodium hydrate, for example to a reaction which just gives a fine pink with coralline (rosolic acid) paper. Of this medium place 5 c.c. in a cordite tube and add to it 0·5 c.c. of 0·01 per cent. solution of phenol red. Note the tint as seen in the comparator with a tube of water superimposed, and then gradually run in the $\frac{N}{20}$ NaOH solution till a tint intermediate between the tints of the two standard tubes is obtained. Repeat the process and take the mean of the two observations. Let the number of c c. of $\frac{N}{20}$ NaOH solution $= X$; then $\frac{X}{20}$ = the number of c c of a normal NaOH solution, and $\frac{X}{20} \times \frac{1000}{5}$ = the number of c.c. of normal NaOH necessary to bring a litre of medium to the required reaction, namely, $P_H = 7{\cdot}5$.

The description given applies to a fluid medium, such as bouillon. It is best to sterilise the medium before standardisation, and after the reaction has been adjusted, subsequent sterilisation should be carried out at 100° C. Even in this case heating tends to raise somewhat the hydrogen-ion concentration, and a tube of the final product should be tested by adding the indicator. Gelatin may be

treated in a similar way to that described, the medium being first liquefied and kept at a temperature of about 30° C.

In the case of agar it is troublesome to carry out the method as directed, owing to the temperature necessary to have the medium liquefied. The procedure usually followed is to bring the bouillon to the required reaction and then to add the agar and liquefy at 100° C. The agar should be previously brought to a fine state of division and well washed in water; if this is done the agar has usually only a trifling effect on the reaction. Here, again, a test of the reaction of the final product should always be made. In carrying out all these procedures, practice is necessary for the attainment of satisfactory results. As a matter of fact, it is our experience that for all ordinary purposes Eyre's method as described above is quite satisfactory.

1 (*b*). **Glucose Broth.**—To the other constituents of bouillon or digest broth there is added 1 or 2 per cent. of glucose. The steps in the preparation are the same.

1 (*c*). **Glycerin Broth.**—Add 1½ to 6 per cent. of glycerin (sp. grav. 1·25) to digest broth or to ordinary broth *after filtration*. This medium is especially used for growing the tubercle bacillus when the products of the growth of the latter are required.

2. **Gelatin Media.**—These are simply the above broths, with gelatin added as a solidifying body.

2 (*a*). **Peptone Gelatin :—**

Meat extract	1000 c.c.	or digest broth 1000 c.c.
Sodium chloride	5 grams	
Peptone	10 ,,	
Gelatin	100–150 ,,	

(The "gold label" gelatin of Coignet et Cie, Paris, is the best.) The sheets of gelatin are rolled up and added with the other constituents to the extract; the mixture is allowed to stand at room temperature for fifteen minutes and is then thoroughly melted in the "Koch" (about half an hour's heating suffices). The fluid medium is then well agitated and is rendered slightly alkaline, and filtered through filter paper. At this state it is practically always necessary to clear the medium. This is done by first cooling to 50° C., then adding the white of one egg, slightly beaten up, to each 1000 c.c. of medium, along with a few pieces of cotton or glass wool, and then boiling in the Koch for one and a half hours. The resulting coagulum carries down any particles which cause turbidity. Thereafter the medium is filtered through Chardin's paper, care being taken that the contents of the flask are not shaken up. As the medium must not be allowed to solidify during the process, it must be kept warm. This is effected by putting the flask and funnel into a tall Koch's steriliser, in which case the funnel must be supported on a tripod or diaphragm, as there is great danger of the neck of the flask breaking if it has to support the funnel and its contents. The filtration may also be carried out in a funnel

with water-jacket which is heated, as shown in Fig. 8. In either case it is advisable to lay a glass plate over the filter funnel to prevent evaporation or condensation water dropping into the medium. A litre flask of the finished product ought to be quite transparent. The flask containing it is then plugged with cotton wool and sterilised, best by method B (2), p. 35. Too much boiling, or boiling at too high a temperature, as has been said, causes a gelatin medium to lose its property of solidification. The exact percentage of gelatin used in its preparation depends on the temperature at which growth is to take place. Its firmness is its most valuable characteristic, and to maintain this in hot summer weather 15 parts per 100 are necessary. But if the gelatin be too stiff, it will split when punctured by the platinum needle used in inoculating it with a bacterial growth; 15 per cent. gelatin melts at about 24° C. For ordinary use in British laboratories 10–12 per cent. gelatin is a sufficient strength.

2 (*b*). **Glucose Gelatin.**—The constituents and mode of preparation are the same as 2 (*a*), with the addition of 1 to 2 per cent. of glucose before sterilisation. This medium is used for growing anaerobic organisms at the ordinary temperatures.

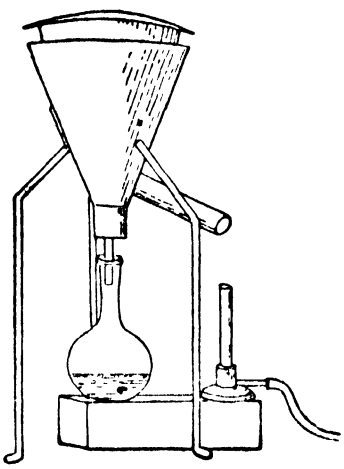


FIG. 8.—Hot-water funnel.

(3). **Agar Media (French, "gélose").** The disadvantage of gelatin is that at the blood temperature (38° C.), at which most pathogenic organisms grow best, it is liquid. To get a medium which will be solid at this temperature, agar is used as the stiffening agent instead of gelatin. Unlike the latter, which is a protein, agar is a carbohydrate. It is derived from the stems of various seaweeds growing in the Chinese seas, commercially classed together as "Ceylon Moss." For bacteriological purposes the dried stems of the seaweed may be used, but there is in the market a purified product in the form of fibre, which is preferable.

3 (*a*). **"Ordinary" Agar.**—This has the following composition :—

Meat extract	1000 c.c.	} or digest broth 1000 c.c.
Sodium chloride	5 grams	
Peptone	10 ,,	
Agar	15 ,,	

Tear up the agar into fine fragments, add to the other ingredients, and allow to stand for a quarter of an hour. Then boil gently in a "Koch" for one to two hours, or autoclave at 115° C. for thirty minutes, till the agar is thoroughly melted. Clear as in the case of gelatin. Render slightly alkaline with sodium hydrate solution, and if necessary make up to original volume with distilled water.

and filter through Chardin's paper as in the case of gelatin. If a slight degree of turbidity may be tolerated, it is sufficient to filter through a felt bag or jelly strainer. Plug the flask containing the filtrate, and sterilise either in autoclave for fifteen minutes or in Koch's steriliser for twenty minutes on each of three successive days. Agar melts just below 100° C., and on cooling solidifies at about 39° C.

3 (*b*). **Glycerin Agar.**—To 3 (*a*) after filtration add 1½–6 per cent. of glycerin and sterilise as above. This is used especially for growing the tubercle bacillus.

3 (*c*). **Glucose Agar.**—Prepare as in 3 (*a*), but add after filtration 1 to 2 per cent. of glucose, or, better still, a corresponding amount of a 10 per cent. sterile solution of glucose. This medium is used for the culture of anaerobic organisms at temperatures above the melting-point of gelatin. For the growth of the tetanus bacillus a specially suitable medium is composed of meat extract with 2 per cent. agar, 2 per cent. peptone, and 0·5 per cent. alkaline sodium phosphate added, and made faintly alkaline to phenol-phthalein; 1 per cent. of glucose is added as above.

Hartley's Broth

This is a medium very suitable for the production of diphtheria toxin. One hundred and fifty grams of minced horse muscle are mixed with 250 c.c. tap water and heated to 80° C. in a steamer: 250 c.c. of 0·8 per cent. sodium carbonate solution (the anhydrous salt) are then added, and the mixture cooled to 45° C., after which 5 c.c. of chloroform and 5 c.c. of pancreatic extract [1] (Cole and Onslow) are added (the amount of pancreatic extract required varies with different preparations). The mixture is incubated at 37° C. for six hours, the vessel being shaken at frequent intervals. Forty c.c. of normal hydrochloric acid are then added, and the mixture heated in a steamer for half an hour, then cooled and filtered. The reaction of the filtrate is adjusted to $P_H = 8$, and the medium distributed into containers. For the sterilisation of small quantities (100 c.c. medium in half-litre bottles) free steam is passed through the autoclave for one hour, then the pressure is raised slowly to 10 lb. and the steam turned off. For larger quantities (1 litre of medium in double Winchester quart bottles) the same method of sterilisation is adopted, except that the pressure is maintained at 10 lb. for half an hour.

[1] The fresh pancreas of a pig is freed from fat as far as possible, and weighed. It is minced finely and three times its weight of distilled water and its own weight of strong alcohol are added. Shake the mixture well in a large bottle and allow it to stand for three days at room temperature, shaking the bottle occasionally. Strain through muslin and filter through a large folded filter paper. The filtrate, which comes through very slowly, is measured and treated with 1 c.c. of concentrated hydrochloric acid for every litre. This causes the appearance of a cloudy precipitate which settles in a few days and can be filtered off. The fluid keeps for an indefinite period if stoppered, without any additional antiseptic. If desired at once, the extract may be used before adding hydrochloric acid, the function of which is to retard the slow auto-destruction of the trypsin.

Robertson's Bullock's Heart Medium

This medium was introduced especially for the cultivation of anaerobes, and is made as follows: 1 lb. of bullock's heart is minced; 500 c.c. of tap water are added and the mixture is heated slowly in the Koch for several hours so as to cook the meat thoroughly; normal sodium hydrate is added until the reaction is alkaline to litmus; it is then strained through gauze and the fluid is thoroughly squeezed out and preserved. The residue is now minced again as finely as possible. The fluid and the mince are autoclaved in separate flasks for thirty minutes at 115° C. When required for use sterile test-tubes are filled to one quarter with the mince, and then the fluid is added till the tube is half full. Sterilise by autoclaving at 115° C. for twenty minutes on two successive days. For growing cultures of anaerobes in the ordinary atmosphere the medium should be freshly prepared, or if it has been tubed for some time it should then be heated for one hour in the Koch.

Peptone Water

A simple solution of peptone constitutes a suitable culture medium for many bacteria. The peptone in the proportion of 1 to 2 per cent., along with 0·5 per cent. NaCl, is dissolved in distilled water by heating. The fluid is then filtered, placed in tubes, and sterilised. The reaction is usually distinctly alkaline, which condition is suitable for most purposes. For special purposes the reaction may be standardised. In such a solution the cholera vibrio grows with remarkable rapidity. It is also much used for testing the formation of indol by bacteria; and by the addition of one of the sugars to it the fermentative powers of an organism may be tested (p. 62), an indicator being added to show any change in reaction.

It is to be noted that, in general, when preparing media the minimum amount of heating should be used, as prolonged exposure to high temperatures damages their nutritive properties. These bouillon, gelatin, and agar preparations constitute the most frequently used media. Growths in bouillon do not usually show any characteristic appearances which facilitate classification, but such a medium is of great use in investigating the soluble toxic products of bacteria. The most characteristic developments of organisms take place on the gelatin media. These have, however, the disadvantage of not being available when growth is to take place at any temperature above 24° C. For higher temperatures agar must be employed. Agar is, however, never so transparent. Though quite clear when fluid, on solidifying it always becomes slightly opaque. Further, growths upon it are never so characteristic as those on gelatin. It is not liquefied by any of the pathogenic bacteria, whereas some organisms, by their growth, liquefy gelatin and others do not—a fact of prime importance.

Special Culture Media

Very many different media have been used either where special difficulty is experienced in getting an organism to grow, or where some special growth characteristic is to be studied. It is impossible to do more than give the chief of these.

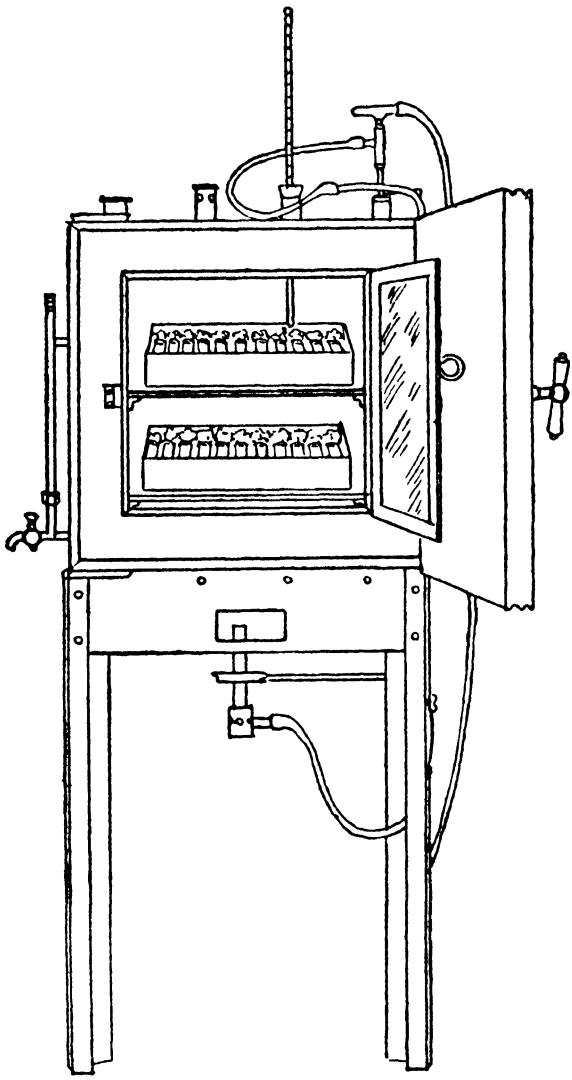


Fig. 9.—Blood serum inspissator.

Serum and Blood Media

Inspissated Blood Serum. — Koch introduced this medium for the cultivation of the tubercle bacillus, and in order to obtain it in a comparatively clear state, adopted the method of inspissation at 65° C. after sterilising by the intermittent method at low temperature—B (4) (p. 37). "Inspissation" is an initial stage of coagulation, and is effected by keeping the serum at 65° C. till it stiffens. This temperature is just below the coagulation point of the serum. The more slowly inspissation is performed the clearer will be the serum. The apparatus used for the purpose is one of the various forms of serum steriliser (*e.g.* Fig. 9), generally a chamber with water-jacket heated with a Bunsen below. The temperature is controlled by a gas regulator, and such an apparatus can, by altering the temperature, be used either for sterilisation or inspissation. The procedure is somewhat tedious, and for all ordinary purposes opaque coagulated serum, sterilised by the usual methods, can be substituted.

Coagulated Serum.—A sufficient quantity of serum is placed in a series of sterile test-tubes: these are then placed in a sloped tray, and heated in the Koch at 80° C. for an hour. If the process of heating is carried out too quickly, bubbles of gas are apt to form and to tear up the surface of the medium. This can be avoided if

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the proportion of about 5 drops to 5 c.c. of agar ; the blood is added to the melted agar as in Wertheim's medium. W. B. M. Martin recommended the substitution of di-sodium phosphate (0·5 per cent.) for sodium chloride in the preparation of the agar, and used fluid human serum sterilised at 57° C. in place of blood. He also found that the same agar medium allowed to solidify and then smeared on the surface with a drop or two of human serum or blood gave excellent results.

Any of these media may be used for plate cultures, the agar being melted and cooled to 45° C. as for agar plates ; the serum or blood is then added, and the mixture is poured out in Petri dishes.

Medium for Meningococcus. — *Trypagar.*—Gordon has introduced this medium for the cultivation of the meningococcus. It is prepared as follows :

1. Take 50 grams of pea flour (ordinary Pearce Duff's) and add to 1 litre of distilled water with 100 grams of salt. Mix and steam for half an hour, stirring occasionally ; allow to settle, and filter, then sterilise and label " Saline Pea Extract." This pea extract should preferably be freshly made for each batch of agar.

2. Take some fresh bullocks' hearts, free from fat and vessels, mince the meat very finely, and weigh. To each ½ kilo add 1 litre of water, and make faintly alkaline to litmus with 20 per cent. caustic potash solution. Heat this slowly to 75°–80° C. for five minutes. Cool to 37° C., and add 1 per cent. of Liquor Trypsinæ Co. (Allen & Hanburys), and keep it at 37° C. for two and a half to three hours. When trypsinising is finished, test for peptone with copper sulphate and caustic potash as below, then render slightly acid with glacial acetic acid, and bring slowly to the boil for a quarter of an hour. Leave overnight in a cool place, and siphon off the clear liquid in the morning. Make this faintly alkaline to litmus, and sterilise in the autoclave at 118° C for one hour on each of two days (if not to be used at once) The result is trypsinised broth.

Method.—Take a measured quantity of the trypsinised broth, add 2 per cent. of agar fibre (see below for preparation), and 0·215 gram of calcium chloride per litre. Autoclave at 118° C. for three-quarters of an hour to dissolve the agar. Mix together in an urn or saucepan ; titrate with $\frac{N}{10}$ caustic soda while boiling, using phenolphthalein as the indicator, and add the necessary amount of normal caustic potash to give an absolutely neutral reaction. Cool to 60° C., add white of egg (2 to a litre) beaten up with the crushed shells, autoclave again at 118° C. for seventy-five minutes (or in the steamer for two hours). Filter, add to the filtrate 5 per cent. of the sterile pea extract, and sterilise in the ordinary way. For use, a small quantity of sterile rabbit's blood or serum—5 c.c. to 200 c.c. of medium—is added to the medium in the melted state at 50° C. before being poured into capsules, or a drop or two of serum may be spread with a glass rod over the surface of the medium after it has solidified.

Preparation of Fibre Agar. — Weigh out the required quantity, cut up small with scissors, place in a large flask or enamel pail, and wash twice quickly in water. Drain thoroughly ; add water just to cover, and put in glacial acetic acid, 2·5 c.c. per litre of water. Mix thoroughly and leave for a quarter of an hour. Pour off the

liquid and wash *thoroughly* four or five times to make sure that all the acetic acid is washed out. Drain carefully, and use as above.

Biuret Reaction for Peptone.—Take 5 c.c. of broth, add 1 c.c. of 5 per cent. solution of copper sulphate. Mix, and then add 5 c.c. normal caustic potash. A true pink colour indicates that trypsinisation is sufficient ; a bluish purple shade, that it is incomplete.

Bordet and Gengou's Medium for Bacillus of Whooping-Cough.—An extract of potato is first prepared by adding two parts of water containing 4 per cent. of glycerin to one part of potato chips ; the mixture is then boiled and the fluid is separated off. An agar medium is then prepared of the following composition : potato extract, 50 c.c. ; 0·6 per cent. solution of sodium chloride, 150 c c. ; and agar, 5 grams Of this medium, 2–3 c.c. are placed in each of a series of sterile test-tubes, and then to each there is added, by the method described above (p. 53) an equal part of defibrinated rabbit's (or better, human) blood, obtained by aseptic methods. The mixture is then allowed to solidify in the sloped position. This medium is also very suitable for the growth of the gonococcus, meningococcus, and influenza bacillus.

Blood-Alkali Agar (Dieudonné). — This medium, introduced for the culture of the cholera vibrio, for which purpose it has been found extremely suitable, has the property of inhibiting the growth of most of the intestinal bacteria ; for example, the B. coli does not grow on it, or does so very slightly. A blood-alkali solution is prepared by adding equal parts of defibrinated ox blood and of normal caustic soda solution ; the solution may then be heated in the Koch for thirty minutes ; it keeps for two months. Of this solution three parts are added to seven parts of ordinary agar medium or digest agar rendered neutral to litmus, and the mixture is poured into plates which, after drying, should be kept at room temperature for twenty-four hours before they are inoculated.

Egg Media

Media containing either the yolk, or both the yolk and the white of egg, have been used for the culture of the tubercle bacillus by Dorset and others. The following will be found very suitable :

Egg Medium.—Break into a sterile bowl fresh eggs, which have been washed in soap and water and then dried, and beat with a sterile knife to mix the yolks and whites. Strain the mixture through sterile cheese cloth (several layers of the cloth are stretched over a filter funnel and sterilised in the Koch) ; and to every 75 c.c. of egg (about two eggs) add 25 c.c. of sterile twenty-one days' heart digest broth and 1 c.c. of 1 per cent. watery solution of crystal violet. Fill the medium into sterile test-tubes, avoiding the formation of air bells, and coagulate by heating in the Koch at 80° C. (the tubes should be placed high up in the steriliser, in a sloping position). Sterilise by heating in the Koch at 80° C. for two hours on three successive days. The medium keeps for several months provided drying does not occur. If the surface of the medium has become dry before the inoculation of a tube, two drops of sterilised water are placed on the surface. The inoculation material

is well rubbed over the surface of the medium, the tubes are sealed with a few drops of melted paraffin wax on the top of the plug and are incubated in the sloped position.

Glycerin Egg Medium is prepared as above by using heart digest broth with the addition of 6 per cent. of glycerin.

Alkaline Egg Medium.—Beat up the yolk of one egg with the whites of two, then add 6 c.c. $\frac{N}{1}$ NaOH, and finally add slowly 500 c.c. tap water. Heat the mixture very slowly to 95° C. and keep it at this temperature for not less than one hour, then filter through cotton wool and muslin, tube and autoclave at 115° C. for twenty minutes. When cool, one part of the fluid may be added to five parts of broth.

Potatoes as Culture Material

Large potatoes are selected ; the surface is cleansed by scrubbing in running water and cylinders are cut with an apple corer or large cork borer (Fig. 10). Wash in running water for two hours, to remove excess of starch. Divide the cylinders by a diagonal cut and place each half thus obtained in a sterile test-tube, the broad end of the cylinder resting on wet cotton-wool or in a tube of the form shown in Fig. 11. Fill the tubes with sterile water and boil in the Koch for half an hour. Pour off the water and autoclave at 115° C. for twenty minutes.



FIG. 10.—Cylinder of potato cut obliquely.

Glycerin Potato Medium.—The method of preparation is the same as in ordinary potato medium, except that 6 per cent. glycerin is used in place of the sterile water for filling the tubes.

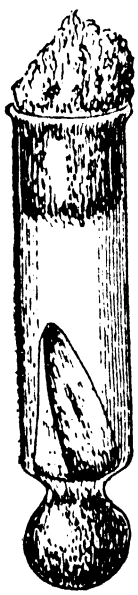


FIG. 11.—Ehrlich's tube, containing piece of potato.

Alkaline Potato Medium.—After boiling in the Koch as described for ordinary potato medium, the water is poured off and the tubes filled with sterile 0·7 per cent. sodium bicarbonate solution ; the subsequent treatment is the same as in ordinary potato medium.

Potatoes ought not to be prepared long before being used, as the surface is apt to become dry and discoloured. It is well to take the reaction of the potato with litmus before sterilisation, as this varies ; normally in young potatoes it is weakly acid. The reaction of the potato may be more accurately estimated by steeping a given weight of potato slices for some time in a known quantity of distilled water, and then estimating the reaction of the water by phenolphthalein. The required degree of acidity or alkalinity is obtained by adding the necessary quantity of HCl or NaOH solution (p. 43), and steeping again. The water is then poured off and the potatoes placed in tubes. Potatoes before being inoculated ought always to be incubated at 37° C. for a night, to make sure that the sterilisation has been successful.

Milk as a Culture Medium

This is a convenient medium for observing the effects of bacterial growth, in coagulating the soluble albumin, and in fermenting the lactose. It is prepared as follows : Boil well-skimmed milk in the Koch for ten minutes ; allow to cool and filter through Chardin's filter paper. Add 6 per cent. litmus solution (Kubel-Tiemann's) and adjust the reaction. Fill into test-tubes and sterilise for twenty minutes on three successive days. This is *litmus milk*. The litmus may be omitted. The reaction of fresh milk is alkaline. If great accuracy is necessary, any required degree of reaction may be obtained by the titration method.

Bread Paste

This is useful for growing torulæ, moulds, etc Some ordinary bread is cut into slices, and then dried in an oven till it is so dry that it can be pounded to a fine powder in a mortar, or rubbed down with the fingers and passed through a sieve. Some 100 c.c. flasks are washed, dried, and sterilised, and a layer of the powder half an inch thick placed on the bottom. Distilled water, sufficient to cover the whole of it, is then run in with a pipette held close to the surface of the bread, and, the cotton-wool plugs being replaced, the flasks are sterilised in the Koch's steriliser by method B (2). The reaction is slightly acid.

Media used for Separating the Members of Bacterial Groups

A great number of media have been devised for use in differentiating the members of the coli-typhoid and other bacterial groups. The general feature of these media is that they contain certain substances, often sugars, which tend to bring out the special characters of the organism under investigation. Sometimes also substances are present which inhibit the growth of bacteria other than those belonging to the group. The following are the media which here deserve most attention :

MacConkey's Bile-Salt Media.—These media were introduced for the purpose of differentiating the intestinal bacteria, and have been extensively used for the study of the B. coli, B. typhosus, B. dysenteriæ, etc. The characteristic ingredients are bile salts and various sugars. The stock solution is the following : Commercial sodium taurocholate, 0·25–0·5 gram (the exact quantity depends on the specimen of bile salts ; in some cases the larger amount may be inhibitory) ; sugar-free peptone, 2 grams ; tap water, 100 c.c. (if distilled water be used, 0·03 per cent. of calcium chloride should be added). The solution is steamed for two hours, filtered when hot, allowed to stand for twenty-four hours or till sedimentation has occurred, and filtered again. For a liquid medium there is added to this 0·25–0·5 per cent. of a freshly prepared 1 per cent. solution of neutral red and the sugar—when glucose, dulcitol, or adonitol is used, 0·5 per cent. is added, in the case of other sugars

1 per cent. The fluid is distributed in Durham's fermentation tubes and sterilised in the steamer for ten minutes on two successive days, care being taken not to overheat the medium.

For bile-salt agar 1·5 to 2 per cent. agar is dissolved in the stock solution in the autoclave, if necessary cleared with white of egg and filtered. Neutral red and 1 per cent. lactose are added, as in the case of the liquid medium (for special purposes other sugars may be substituted for lactose). It is well to sterilise it in flasks containing 80 c.c., this being an amount sufficient for three large Petri capsules. When this medium is used for examining urine or fæces, plates are inoculated (see p. 73) ; for its use in water examinations, see p. 73.

When growth of a bacterium producing acid and gas occurs in neutral-red fluid media the latter turns a rose colour, and gas appears in the Durham's tube. Sometimes a fluorescent appearance is also observed, the significance of which will be discussed in the chapter on B. coli. With the neutral-red solid media the colonies of any organism giving rise to acid will be of a rose-red colour. Litmus may be used instead of neutral red.

Endo's Medium.—

Ordinary meat extract . . .	1000 c.c.
Peptone (sugar-free) . . .	10 grams.
Sodium chloride . . .	5 ,,
Agar-agar fibre cut into small pieces	20 ,,

Mix the ingredients and heat in the autoclave at 115° C. for half an hour ; render alkaline by the addition of 10 c.c of 10 per cent. sodium carbonate. Cool to 50° C. and add the white of an egg. Boil in the Koch for one and a half to two hours ; filter while hot through Chardin's paper. To each 100 c c. of filtrate add 1 gram lactose, 0·05 c.c. of filtered saturated alcoholic solution of basic fuchsin, and 2·5 c.c of freshly prepared 10 per cent. sodium sulphite solution. Sterilise in the Koch for twenty minutes on three successive days. When the medium is hot it appears pink in colour, when cold it is almost colourless. (Should the medium be pink when cold the amount of sodium sulphite present is insufficient.) The medium must be kept in the dark.

These media in the hands of a worker accustomed to their use will yield good results. MacConkey's medium is that most used by British workers, and it has the merit of being easily prepared. As the result of a considerable experience we have found it most useful and reliable.

Brilliant Green Enrichment Method (Browning, Gilmour, and Mackie).—In this method advantage is taken of the fact that brilliant green has a greater inhibitory effect on B. coli generally than on B. typhosus and the paratyphoid group of bacilli. The amount of the dye necessary to bring about the desired result is not a fixed quantity in each case, as it depends on the number of organisms in the fæces and also on the organic matter. A number of dilutions of the dye are therefore used. Tubes of peptone water neutral to litmus paper (peptone 2 per cent. and sodium chloride 0·5 per cent.) each containing 10 c.c. are prepared. The brilliant

green sulphate (zinc free) is used as a 1 : 10,000 solution in distilled water. To three tubes of peptone water, 0·7, 0·4, and 0·25 c.c. of the brilliant green solution are added in series. Each tube is then inoculated with several large loopfuls of fæces (where the fæces are not fluid a thick suspension is made in sterile saline), and the tubes are incubated at 37° C. for twelve to twenty-four hours. At the end of this time a loopful is taken from each tube and strokes are made on plates of MacConkey's medium—three strokes with each loopful. Two plates will be sufficient for the strokes from all the dilutions. After incubation for another twenty-four hours the plates are examined for typhoid colonies; often a pure culture is obtained from one of the dilutions When the bacilli are scanty the results yielded by the method are often remarkable. The method is not suitable for the isolation of dysentery bacilli.

Whilst B. coli generally is inhibited by the brilliant green, Browning and his co-workers have found that some strains, especially the inosite-fermenters, *e.g.* B. lactis aerogenes, are equally resistant with B. typhosus, but, on the other hand, are much less resistant to telluric acid They therefore recommend that 0·4 c.c. of a 1 : 1000 solution of telluric acid be added to the tubes of peptone water along with the varying amounts of brilliant green.

Whilst a number of tubes, as above described, are essential for the best results, a one-tube method may often be used with success. In this case 0·5 c c. of the 1 : 10,000 solution of brilliant green is the optimum quantity. This is specially successful with paratyphoid B.

Methods have also been devised, on the same principle as the above, for inhibiting the growth of various organisms present along with B. diphtheriæ, and thus aiding the isolation of the latter. We give the following:

Conradi and Troch's Method for isolating the B. diphtheriæ.—This medium is made by mixing 1000 c.c. water, 10 grams Lemco, 5 grams sodium chloride, 20 grams Witte's peptone, and 6 grams calcium bimalicum, steaming for half an hour and filtering. To this slightly acid fluid 1 per cent. of glucose is added and one part is mixed with three parts fresh ox serum. To each 100 c.c. of the bouillon-serum medium 2 c.c. of a 1 per cent. solution of potassium telluricum is added. The finished medium is distributed in Petri capsules and coagulated by a quarter of an hour's exposure to 85° C. A tube of ordinary Löffler's serum is inoculated with the material to be examined for the diphtheria bacillus and incubated for three hours. The surface is then scraped and two plates of the special medium are inoculated, and incubated for twenty hours. Any diphtheria colonies present are a deep black from a reduction of the dioxide of tellurium; pseudo-diphtheria colonies show yellow-grey or greyish-black.

Smith's Method.—The following medium, containing telluric acid, has been devised by J. F. Smith; it gives excellent results. It has the composition:

Peptone-water agar (neutral to litmus) . .	100 c.c.
Sheep's serum (sterilised at 57° C.) . . .	5 ,,
1 per cent. telluric acid solution in distilled water	0·9 ,,

The serum is added to the melted agar at a temperature of 50° C. On this medium the diphtheria bacillus forms large white colonies after incubation for twenty-four hours. The growth of many organisms is inhibited.

Media for growing Trichophyta, Moulds, etc.

1. *Beer Wort Agar.*—Take beer wort as obtainable from the brewery and dilute it till it has a s.g. of 1100. Add 1·5 per cent. of powdered agar, and heat in the Koch till it is dissolved (usually about two hours are necessary). Filter rapidly and fill into tubes. Sterilise in the Koch for twenty minutes on three successive days. If the medium is heated too long it loses the capacity of solidifying.

2 *Sabouraud's Media.*—Sabouraud recommends the following media, the first being that most frequently used :

(1)	Pure tap water	1000 c.c.
	Maltose (" brute de Chanut ") .	40 grams.
	Peptone (" granulée de Chassaing ")	10 ,,
	Agar	18 ,,
(2)	Pure tap water	1000 c.c.
	Glucose (" massée de Chanut ") .	40 grams.
	Peptone (" granulée de Chassaing ")	10 ,,
	Agar	18 ,,

In order to secure uniformity of results over as long a series of observations as possible, it is advisable to make up these media in large quantities, say three litres at a time in a five-litre flask. The agar is put to soak in the water for an hour, the other ingredients are added and dissolved by gradually heating to 120° C. in an autoclave The medium is then thoroughly mixed by stirring and rapidly filtered through *papier Chardin* (Cogit, 36 Boulevard Saint Michel, Paris). For this purpose, Sabouraud recommends that ten 500 c.c. flasks should be fitted with funnels and filtration simultaneously carried on in the whole series ; whenever in any one of the flasks the filtrate begins to pass only in drops, a new filter paper is substituted. In this way the three litres of medium can be filtered in a few minutes. We have found that the procedure can be simplified without apparently affecting the efficiency of the medium, by dissolving the agar and sugar in one flask, and the peptone in another. The contents of each are filtered and the two filtrates are then mixed ; in this procedure only two or three filter papers are required for the rapid filtration of a large quantity of the agar and sugar moiety. If filtration in a number of flasks is practised, the contents of all are mixed and then distributed in 6 × ⅝ inch test-tubes (plugged with non-absorbent cotton) and sterilised by one exposure in the autoclave at 120° C.—the temperature being very gradually raised. These tubes are used for the primary inoculations, and during incubation, which is necessarily prolonged and usually carried out at 22° C., should be placed in a covered glass jar, the lid of which is kept slightly raised at one side with a pad of wool to permit the access of a certain amount of air—by this device undue drying of the medium is at the same time prevented ; the inoculated tubes should not be covered with rubber caps. The study of the characters of the large colonies of trichophyta, etc., is

best carried out with media distributed in 250 or even 500 c.c. Enlenmeyer flasks in which the requisite surface of medium with a suitably moist atmosphere is obtained.

The Observation of Bacterial Fermentation of Sugars, etc.—The capacity of certain species of bacteria to ferment sugars constitutes an important biological property. As a result of this action the fermentable substance is broken down and acid products are formed, and also frequently gases. Besides sugars, closely allied bodies which are alcohols with large molecules may be broken down by bacterial action, and these have been used for differentiating the properties of allied bacteria. Similarly glucosides (which are combinations of glucose with other substances) and other substances (*e.g.* inosite) have also been used. The following fermentable substances are commonly employed:

Monosaccharides.—*Pentoses*—arabinose (obtained from gum arabic), xylose (from wood), and rhamnose (which is really a methylpentose). *Hexoses*—glucose (dextrose), lævulose, mannose (from the vegetable ivory nut), and galactose (a hydrolytic derivative of lactose).

Disaccharides.—Lactose, saccharose, maltose.

Polysaccharides.—Starch, raffinose, inulin (from dahlia roots), dextrin, arabin, glycogen, cellulose.

Alcohols.—Glycerol, mannitol, dulcitol, sorbitol, adonitol, erythritol.

Glucosides.—Salicin, coniferin.

The end products of bacterial fermentations may be various. They may be alcohols, acids, or gaseous bodies (chiefly carbon dioxide, hydrogen, and methane). For the estimation of the first groups complicated chemical procedures may be necessary. The tests usually employed for the detection of ordinary fermentative processes depend on two kinds of changes, namely: (*a*) the formation of acids, and (*b*) the evolution of gases. Generally speaking, these tests are reliable, and the methods to be pursued are simple. Besides such gases as those named, some organisms give rise to sulphuretted hydrogen by breaking up the protein. The formation of this gas can be detected by the blackening of lead acetate when it is added to the gas-containing medium.

In testing the effect of a bacterium on a given sugar it is essential that this sugar alone be present; the basis of the medium ought therefore to be either peptone solution or casein digest broth (p. 42). In the case of organisms requiring serum

for their growth Hiss's serum water medium has been used, but is less suitable than peptone water agar to which 5 per cent. of sterile serum has been added. The sugar or other substance is added in the proportion of from a half to 1 per cent. It is preferable that the addition should be made in the form of a sterile 10 per cent. solution in water. If the sugar in solid form be placed in the bouillon and this then sterilised, there is danger that chemical changes may take place in the sugar, in consequence of its being heated in the presence of substances (such as alkalis) which may act deleteriously upon it; in any case sterilisation should not be at a temperature above 100° C.

The *development of an acid reaction* in the medium is demonstrated by the addition of an indicator. In Hiss's serum water media the production of acid also leads to coagulation of the medium. Sometimes acid is formed very slowly from sugars, so that it is well to keep the cultures under observation for several days or even longer.

Acid and gas-formation may be simultaneously tested for, by placing the fluid medium containing the indicator in Durham's tubes.

In all tests in which sugars are used, a control uninoculated tube ought to be incubated along with the bacterial cultures, as changes in reaction sometimes occur spontaneously in media containing unstable sugars. Tests in which sugars are used are best carried out in alkali-free glass tubes.

The capacity of an organism to produce acid may be measured by taking a standard amount of a fluid medium and allowing growth to take place for a standard time, and then adding an amount of, say, decinormal soda solution sufficient to bring the reaction back to that of the original medium.

Indicators

Litmus.—To any of the ordinary media litmus (French, tournesol) may be added to show change in reaction during bacterial growth. The litmus is added, before sterilisation, as a strong watery solution [1]

[1] The litmus solution is made as follows: Solid commercial litmus is digested with pure spirit at 30° C. till on adding fresh alcohol the latter becomes only of a light violet colour. A saturated solution of the residue is then made in distilled water and allowed to stand until the sediment settles or it may be centrifuged. When this is diluted with a little distilled water it is of a violet colour, which further dilution turns to a pure blue. To such a blue solution very weak sulphuric acid (made by adding two drops of dilute sulphuric acid to 200 c.c. water) is added till the blue colour is turned to a wine-red. Then the saturated solution of the dye is added till the blue colour returns.

(*e.g.* the Kubel-Tiemann solution), in sufficient quantity to give the medium a distinctly bluish tint. During the development of an acid reaction the colour changes to a pink, and may subsequently be discharged. The disadvantage of litmus is that the colour change with it is not very sharp.

Neutral-Red.—This dye was introduced by Grünbaum and Hume as an aid in determining the presence or absence of members of the B. coli group, especially in the examination of water. The media found most suitable are agar or bouillon containing 0·5 per cent. of the sugar to be tested, to which 0·5 per cent. of a 1 per cent. watery solution of neutral-red is added. The alkaline medium is of a yellowish brown colour which in the presence of acid passes into a deep rose-red. Sometimes there subsequently occurs a change to a fluorescent green, caused apparently by a change in the composition of the dye, as the fluorescence is not discharged by addition of alkali. (See also p. 400.)

Andrade's Indicator.—An aqueous 0·5 per cent. solution of acid fuchsin is decolorised by the addition of N/1 NaOH. About 16 c.c. are required for 100 c.c. of the dye solution; if the mixture is still red after standing for three hours 1 c.c. of the caustic soda solution should be added further; 1 per cent. of this mixture is added to media. Media with a $P_H = 7{\cdot}2$ are red when hot, but faintly yellow when cold. Acid-formation restores the red colour.

Bromcresol Purple.—The sulphone-phthalein compound bromcresol purple is a useful indicator. It has a purple colour in neutral or alkaline solution, which becomes yellow when the reaction is very slightly acid; hence it is a delicate indicator for detecting acid production: 2 c.c. of a saturated aqueous solution are added to 1000 c.c. of medium.

Hiss's Serum Water Media.—These are composed of one part of ox's serum and three parts of distilled water with 1 per cent. litmus; various sugars in a pure condition are added in the proportion of 1 per cent. The development of acid by fermentation is shown by the alteration of the colour and by coagulation of the medium. These media do not coagulate at 100° C., and thus can be sterilised in the steam steriliser, the same precautions being observed as in the case of other media containing sugars. They have been extensively used in studying the fermentative properties of streptococci and pneumococci, etc. (As controls, cultures of the organisms should be made in the same specimen of serum water medium from which, however, the sugar has been omitted.)

For *the observation of gas-formation* the following methods may be employed:

(1) *Durham's Tube* (Fig. 12, *b*).—A small test-tube is inverted and slipped down into the empty culture tube, which is then plugged and sterilised in the autoclave. The medium, which has been previously sterilised, is then tubed with aseptic precautions and the tubes are finally heated in the Koch for fifteen minutes on each of three successive days. The air remaining in the smaller tube is thereby expelled. The tube is then inoculated with the bacterium to be tested. Any gas developed collects in the upper part of the inner tube. As some of the sugars now used for fermentation tests

are expensive, it is well to arrange the Durham apparatus with very small tubes ; with these a satisfactory result can be obtained with only 1 c.c. of medium.

(2) *The Fermentation Tube* (Fig 12, *c*).—This consists of a tube of the form shown, and the figure also indicates the extent to which it ought to be filled. It is inoculated in the bend with the gas-forming organism, and when growth occurs the gas collects in the upper part of the closed limb, the medium being displaced into the bulb. If the limb be graduated the amount of gas evolved can be measured, and rough chemical tests can be applied, *e.g.* the presence of carbonic acid gas can be tested for by absorbing it with a solution of caustic soda, and that of hydrogen by ignition (see under B. coli).

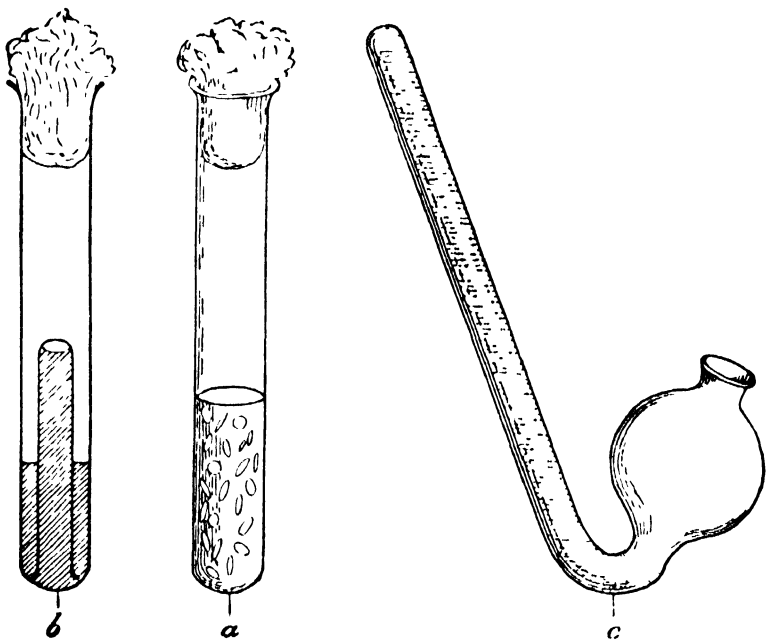


FIG. 12.—Tubes for demonstrating gas-formation by bacteria.

a, tube with "shake" culture
b, Durham's fermentation tube
c, ordinary form of fermentation tube

The Observation of Indol-formation by Bacteria. — The formation of indol from protein by a bacterium sometimes constitutes an important specific characteristic. To observe indol production the bacterium is grown, preferably at incubation temperature, in a fluid medium containing peptone. The latter may either be peptone solution (p. 51) or casein digest broth (p. 42). Any medium containing sugars must be avoided, as the presence of these substances may inhibit the production of indol. Several methods are in use for the detection of this body.

(1) *The Nitroso-indol Method.*—Indol is here recognised by the fact that when it is acted on by nitric acid *in the presence of nitrites,* a nitroso-indol compound is produced, which has a rosy red colour. Some bacteria (*e.g.* the cholera vibrio) produce nitrites as well as indol, but usually in making the test (*e.g.* in the case of B. coli) the nitrites must be added. This is effected by adding to an ordinary tube of medium 1 c.c. of a 0·02 per cent. solution of potassium nitrite, and testing with pure nitric or sulphuric acid. In any case, only a drop of the acid need be added to, say, 10 c.c. of medium. If no result be obtained at once it is well to allow the tube to stand for an hour, as sometimes the reaction is very slowly produced. In many instances incubation at 37° C. for several days may be necessary before the presence of indol is demonstrable. The amount of indol produced by a bacterium seems to vary very much with certain unknown qualities of the peptone. It is well, therefore, to test a series of peptones with an organism (such as the B. coli) known to produce indol, and, noting the sample with which the best reaction is obtained, to reserve it for making media to be used for the detection of this product. This method has for long been felt not to be satisfactory.

(2) *Ehrlich's Rosindol Reaction.*—Böhme showed that for ease of application and delicacy of effect the reaction possesses great advantages. It depends on the fact that paradimethylamidobenzaldehyde unites with indol to form a rosindol body whose colour is readily developed, especially in presence of an oxidising substance such as potassium persulphate ($K_2S_2O_8$). Two solutions are required :

(1)	Paradimethylamidobenzaldehyde . .	4 grams.
	Absolute alcohol (96 per cent.) . .	380 c.c.
	Concentrated hydrochloric acid . .	80 ,,
(2)	Potassium persulphate . .	Saturated watery solution.

To a culture of the organism in 5 c.c. of suitable fluid medium add 1 c.c. of (1) and then 1 c.c. of (2), and shake well ; if indol be present a rose-red colour will appear in a few minutes. Sometimes the rose colour appears on the addition of solution (1), and the addition of a special oxidising agent is unnecessary. The rosindol compound can be separated from the culture by shaking the latter up with amyl alcohol, and MacConkey recommends that this should be done in cases of a doubtful reaction, as sometimes when a faint pink colour appears in the culture tube the extracting alcohol remains colourless, showing that no real reaction has occurred ; chloroform may also be used. Marshall has pointed out that by means of the reaction a quantitative estimate of the amount of indol formation can be obtained. To do this a large culture, say 100 c.c., is distilled, and the colour obtained by applying the test to the distillate in a Nessler's tube is matched against that obtained with different amounts of a standard solution of indol (prepared by dissolving 1 gram indol in 5 c.c. absolute alcohol, and making up to 500 c.c. with distilled water).

The Ehrlich test is from five to ten times more delicate than the ordinary nitroso-indol reaction, and it is of especial value in dealing with organisms of the coli-typhoid group. With strains of B. coli it can often be obtained in from twenty-four to forty-eight hours, but in the case of a negative result a culture of from six to seven

days ought to be used. The reaction is also obtainable with the cholera vibrio, but further investigation is here necessary, as Marshall states that under certain circumstances the nitrites formed by this bacterium may have an inhibitory effect on the production of the rose colour.

The Use of the Ordinary Culture Media

The culture of bacteria is usually carried on in test-tubes conveniently $6 \times \frac{5}{8}$ inches, but for many purposes smaller tubes, $5 \times \frac{1}{2}$ inches, are equally suitable and medium is thus saved. The tubes ought to be very thoroughly washed and dripped, and their mouths plugged with plain cotton wool. They are then sterilised for one hour at 170° C. If the tubes be new, the glass, being usually packed in straw, may be contaminated with the extremely resistant spores of the B. subtilis. Cotton-wool plugs are universally used for protecting the sterile contents of flasks and tubes from contamination with the bacteria of the air. A medium thus protected will remain sterile for years. Whenever a protecting plug is removed for even a short time, the sterility of the contents may be endangered. It is well to place the bouillon, gelatin, and agar media in the test-tubes directly after filtration. The media can then be sterilised in the test-tubes.

In filling tubes, care must be taken to run the liquid down the centre, so that none of it drops on the inside of the upper part of the tube with which the cotton-wool plug will be in contact, otherwise the latter will subsequently stick to the glass and its removal will be difficult. Fig. 13 shows the apparatus which may be used for filling tubes (all the parts should be sterilised before use). It is often convenient to employ instead of the flask a large glass funnel to which the nozzle is attached by rubber tubing ; to prevent contamination from the air the sterile lid of a Petri plate should be laid over the mouth of the funnel. In the case of liquid media, test-tubes are filled about one-third full. With the solid media the amount varies. In the case of gelatin media, tubes filled one-third full and allowed to solidify while standing upright, are those commonly used. With organisms needing an abundant supply of oxygen the best growth takes place on the surface of the medium, and for practical purposes the surface ought thus to be as large as possible. To this end "sloped" agar and gelatin tubes are used (Fig. 14). To prepare these, tubes are filled only about one-sixth full, and after sterilisation are allowed to solidify lying on their sides with their necks supported so that the contents extend 3 to 4 inches

up, giving an oblique surface after solidification. Thus agar is commonly used in such tubes (less frequently gelatin is also "sloped"), and this is the position in which blood serum is inspissated. Tubes, especially those of the less commonly used media, should be placed in large jars provided with stoppers, otherwise the contents are apt to evaporate. A tube of medium which has been inoculated with a bacterium, and on which growth has taken place, is called a "culture." A "pure

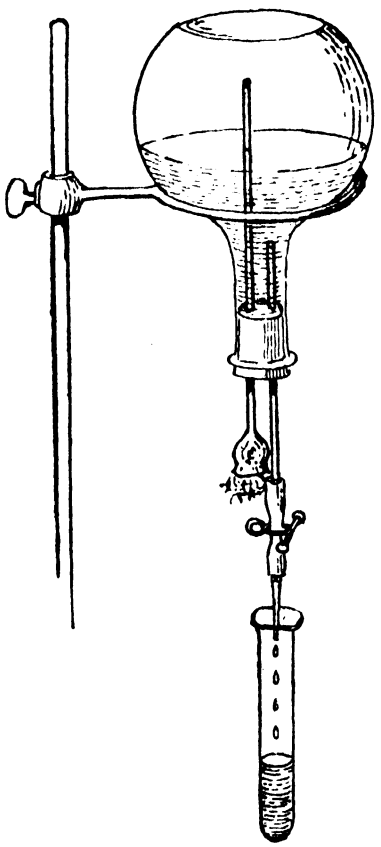


FIG. 13.—Apparatus which may be used for filling tubes.

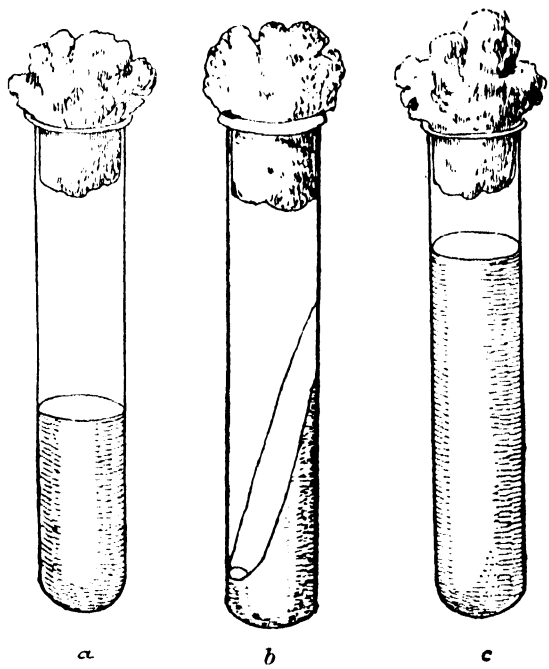


FIG. 14.—Tubes of media.

a. Ordinary upright tube.
b. Sloped tube.
c. "Deep" tube for cultures of anaerobes.

culture" is one in which only one species is present. The methods of obtaining pure cultures will presently be described. When a fresh tube of medium is inoculated from an already existing culture, the resulting growth is said to be a "sub-culture" of the first. Manipulations involving the transference of small portions of growth either from one medium to another, as in the inoculation of tubes, or, as will be seen later, to cover-glasses for microscopic examination, are effected by pieces of platinum wire (Nos. 24 or 27 Birmingham wire gauge—the former

being the thicker) fixed in glass rods 8 inches long, or in aluminium holders. If platinum wire is not available an excellent substitute is found in " resistance wire," No. 25 B.W.G. This is best mounted in an aluminium handle. Every worker should have three wires. Two are 2½ inches long, one of these being straight (Fig. 15, *a*), and the other having a loop turned upon it (Fig. 15, *b*). The latter is referred to as the platinum " loop " and is used for many purposes. " Taking a loopful " is a phrase constantly used. The third wire (Fig. 15, *c*) ought to be 4½ inches long and straight. It is used for making anaerobic cultures. It is also very useful to have at hand a platinum-iridium spud. This consists of a piece of platinum-iridium about 1½ inches long, 2 mm. broad, and of sufficient thickness to give it a firm consistence ; its distal end is expanded into a diamond shape and its proximal is screwed into an aluminium rod (resistance wire

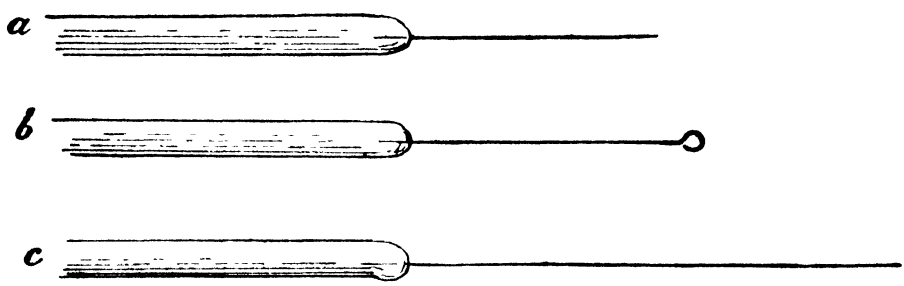


FIG. 15.—Platinum wires in glass handles.

a. Straight needle for ordinary puncture inoculations *b* Platinum "loop." *c* Long needle for inoculating "deep" tubes

may be used). It is very useful for making scrapings from organs and for disintegrating felted bacterial cultures ; in such manipulations the ordinary wire bends too easily.

If a platinum wire heavily charged with bacteria be sterilised in a Bunsen flame it may " spark " and unkilled bacteria may thus fall on the worker's bench. In working with organisms highly pathogenic to man, *e.g.* those of glanders, plague, Malta fever, it is well to substitute for platinum needles glass rods drawn out to capillary diameter, each of which can be destroyed after use. These before use are sterilised by passing through the flame, and when contaminated are dropped into a 1–1000 solution of corrosive sublimate instead of being heated.

Cultures on a solid medium are referred to (1) as " puncture " or " stab " cultures, or (2) as " stroke " or " slant " cultures, according as they are made on medium (1) solidified in the upright position, or (2) sloped.

To make a Subculture on an Agar "Slope" from another Culture —The two tubes are held between the thumb and first two fingers of the left hand, in a slanting position, toward their lower ends, with the sloped surfaces upward. Then with the right hand rotate the cotton-wool stoppers of both tubes, so that they may be readily removable. Take the end of the holder of the looped needle between the thumb and first two fingers of the right hand. Sterilise the wire by holding it vertically in the Bunsen flame. Remove the stopper of the tube from which the inoculation is to be made with the crooked little finger of the right hand and retain it there; flame the mouth of the tube. Pass the needle into the tube and touch the medium to ensure that the wire is cool (if the wire is too hot it will be seen to melt the medium; one must then wait for a few seconds before proceeding). Touch the growth with the wire, which is then withdrawn; remove the stopper from the second tube with the points of the thumb and index finger and retain it there; insert the wire charged with growth and smear lightly the surface of the agar (avoid cutting into the medium). Withdraw the wire and sterilise it in the flame as before; flame the mouths of the tubes and replace the stoppers in their respective tubes.

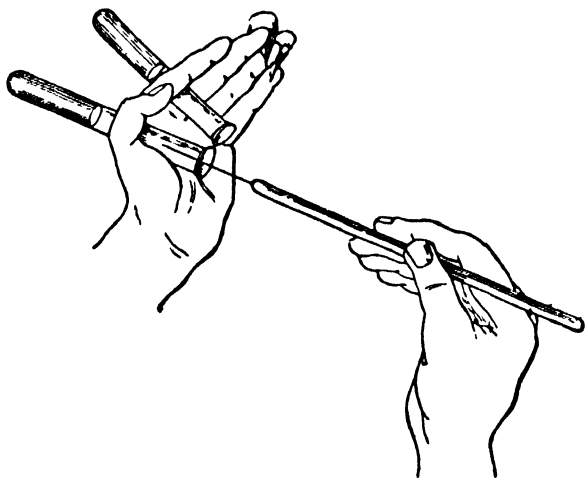


FIG. 16.—Method of making a stab culture.

To make a Stab Culture.—Use the straight wire charged with culture and pass it into the centre of the medium, taking care to withdraw it in the same track so as to avoid splitting the medium. Fig. 16 illustrates the procedure.

To inoculate fluid medium a loopful of culture is deposited on the wall of the tube slightly above the level of the fluid and then the tube is tilted so as to wash the material down. If the inoculum is tenacious, it is rubbed on the wall of the tube, several loopfuls of the contents being incorporated with it by means of the needle, so that a uniform suspension is produced.

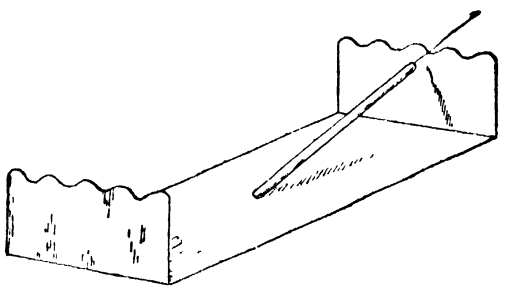


FIG. 17.—Rack for platinum needles.

Hanging-drop Preparations.—It is often necessary to observe micro-organisms alive, either to watch the method and rate of their multiplication, or to investigate whether or not they are motile. This is effected by making hanging-drop cultures. The method in the form to be described is only suitable for aerobes. For this special

slides are used. Two forms are in use, and are shown in Fig. 18. In *A* there is ground out on one surface a hollow having a diameter of about half an inch. That shown in *B* explains itself. The slide to be used and a cover-glass are sterilised by hot air in a Petri's dish, or simply by being heated in a Bunsen and laid in a sterile Petri to cool. In the case of *A*, one or other of two manipulation methods may be employed. (1) If the organism be growing in a liquid culture, a loop of the liquid is placed on the middle of the under surface of the sterile cover-glass, which is held in forceps, the points of which have been sterilised in a Bunsen flame. If the organism be growing in a solid medium, a loopful of sterile bouillon is placed on the cover-glass in the same position, and a *very* small quantity of the culture (picked up with a platinum needle) is rubbed up in the bouillon. The cover is then carefully lowered over the

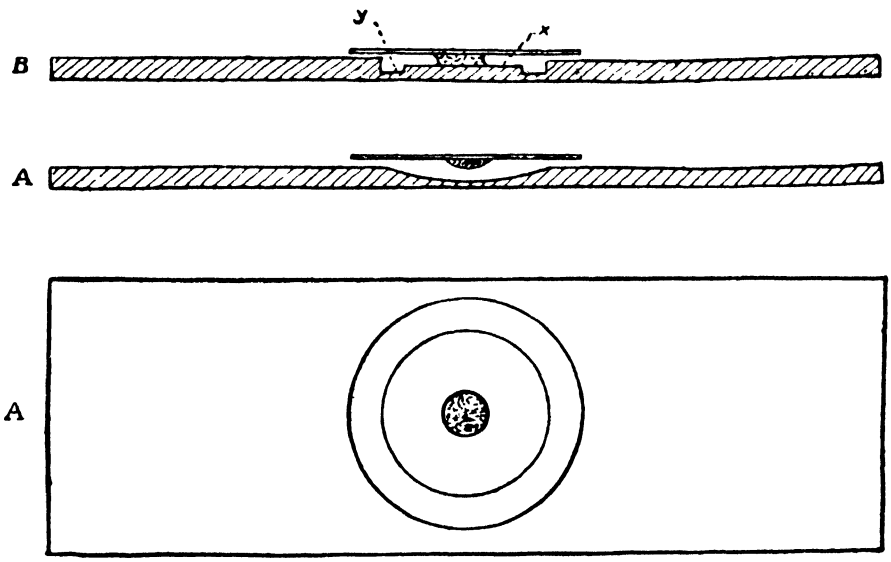


FIG. 18.

A. Hollow-ground slide for hanging-drop cultures, shown in plan and section B. Another form of slide for similar cultures.

cell on the slide, the drop not being allowed to touch the wall or the edge of the cell. The edge of the cover-glass is covered with vaselin, and the preparation is then complete and may be placed under the microscope. If necessary, it may be first incubated and then examined on a warm stage. (2) The sterile cover-glass is placed on a sterile glass plate. The drop is then placed on its *upper* surface, the details being the same as in the last case. The edge of the cell in the slide is then painted with vaselin, and the slide, held with the hollow surface downwards, is lowered on to the cover-glass, to the rim of which it of course adheres. The slide with the cover attached is then quickly turned right side up, and the preparation is complete.

In the case of *B*, the drop of fluid is placed on the centre of the table *x*. The drop must be thick enough to come in contact with the cover-glass when the latter is lowered on the slide, and not large

enough to run over into the surrounding trench *y*. The cover-glass is then lowered on to the drop, and vaseline is painted along the margin of the cover-glass. It is sometimes convenient for the observation of the growth of bacterial colonies or of fungi to make hanging-drop cultures with a solid medium. This can be done by substituting a drop of melted gelatin or agar for bouillon and inoculating the surface after solidification. The method of microscopic examination is described on page 96.

Cultures of Bacteria from Single Cells.—A number of methods have been devised for the purpose of obtaining pure cultures starting from a single organism. Burri's indian ink method consists in emulsifying the organisms in dilute indian ink and depositing by means of a fine pen a number of minute droplets of the mixture on the surface of nutrient medium in a Petri plate. The drops are covered with a cover-slip and are examined under the microscope and those which contain a single bacterium are noted. Time is allowed for multiplication, and a subculture is then made from colonies which have developed from single organisms. With a view to rapid working and to obviating the use of the ink, which is harmful to some bacteria, Ørskov has modified the method as follows: A layer of agar several mm. thick is poured into a flat Petri plate and a suitable loopful of a twelve-hour broth culture, *e.g.* of B. coli, is spread over the medium by means of a bent glass rod. The plate is then placed in the incubator for one hour, at the end of which time multiplication is just beginning in the case of B. coli and the organisms are readily distinguishable under the microscope. A block of agar is excised and its under side placed on a microscopic slide which has previously been sterilised by flaming. The inoculated surface of the agar is examined under the microscope and a field is selected which contains only a single organism. In order to enable the same area to be found again some form of object finder is required for rough orientation; in addition to this, closely spaced crossing lines have previously been cut on the under side of the slide by scratching with a diamond through a drop of immersion oil; by means of these along with an eyepiece micrometer it is possible to register the field exactly, and the position is recorded by careful drawings of the lines. The slide is now placed in a Petri capsule in the incubator, the atmosphere being kept moist by inserting a piece of wet filter paper in the capsule; great care, however, must be taken that the preparation does not become too moist or the agar block will become displaced on the slide. When a colony has developed from the single organism a subculture is made. To do this a piece of fine platinum wire is attached to the front lens of an objective by means of modelling wax, and its point is lowered on to the colony and the organisms which adhere are used to inoculate a tube of broth. (It is necessary to ascertain first of all the exact point in the field which the needle-point impinges on. To do this a small drop of India ink is placed on an agar block similar to that bearing the culture and the needle is lowered on to the agar; the point in the field occupied by the puncture mark is then noted with the microscope and the colony is shifted into this position.) A difficulty in such work is that isolated organisms may fail to yield growths. (For literature on this subject and details, reference must be made to the original paper.)

The Methods of the Separation of Aerobic Organisms. Plate Cultures

The general principle underlying the methods of separation is the distribution of the bacteria in or on one of the solid media so that the colonies formed by the individual organisms are sufficiently far apart to allow their being examined separately. For the purpose, circular shallow glass capsules, each fitted with an overlapping glass cover, are almost universally used; these are known as Petri's dishes or capsules (Fig. 19). The medium, after being melted, is poured into a sterile capsule and allowed to solidify, so as to form a thin layer; in this way the colonies which afterwards grow are readily accessible. In one method the material containing the bacteria is smeared over the surface of the medium after it has solidified in the capsule—"method of successive strokes." In another method the organisms are mixed with the medium when in the melted state, and the mixture is then poured into the capsule and allowed to solidify — "dilution method." The former gives the best results in the case of most pathogenic organisms.

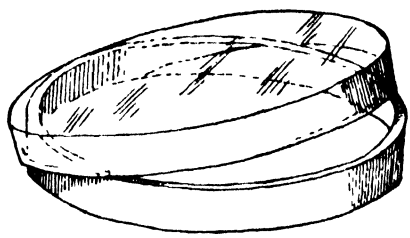


Fig. 19.—Petri's capsule. (Cover shown partially raised.)

The method of successive strokes is the more convenient, and is that used for the separation of typhoid and dysentery bacilli, meningococci, etc.; it is, in fact, capable of almost universal application. The procedure varies according to the material to be examined, but in all cases it is essential to have the surface of the medium dry. This is secured by removing the lid of the capsule when the medium has solidified and covering the plate with sterile paper, after which it is placed in the incubator for half an hour, or drying may be effected more rapidly by warming the open plate quickly over a Bunsen flame (taking care, however, not to melt the medium) and then covering with paper until cooling has occurred. If the organisms are on a swab, say from the naso-pharynx, consectuive strokes are made all over the surface of the medium, always the same portion of the swab being brought into contact with it. In this way the organisms are gradually wiped off the swab, till in the later strokes they may be deposited at sufficiently wide intervals to give separate colonies. Agar plates should always be incubated

in the inverted position to prevent contamination of the surface by condensation water from the lid of the plate. Plates of gelatin, however, are kept with the medium side down, as liquefaction often occurs from the growth of organisms. Sometimes it is advisable to smear several plates consecutively with the same portion of the swab. If the material to be examined is fluid, *e.g.* an emulsion of fæces, the usual method is to place a loopful on the surface of the medium, and then, with a sterile glass rod bent at a right angle, to smear the whole surface. If the organisms are found, on microscopic examination, to be very numerous, say in pus, it will be advisable to dilute with sterile saline before making the smears. The characters of the colonies which appear on the plates can be examined with a hand-lens, magnifying about 6 diameters. In some cases examination under a low-power of the microscope is an advantage; the plate in the inverted position can be put on the stage of the microscope for this purpose. For the culture of special organisms, as afterwards detailed, the agar or other medium is smeared with sterile serum or blood according to the growth requirements of the organism, or the serum or blood is added before the medium is poured.

When making cultures from emulsions of fæces in order to recover organisms of the enterica and dysentery groups, it is important to obtain the largest possible number of isolated colonies. The following method adopted by Mackie is very effective (Fig. 20). A loopful of fluid fæces or of fæcal emulsion is spread on the medium at one side of the plate in the area A; then, without recharging the needle, successive series of parallel strokes are made in different directions (*e.g.* a series in the direction indicated by the lines B, a second series in the direction shown by the lines C, and so on)—the flat part of the loop being throughout kept in contact with the medium.

The principle just described may be applied also to agar in tubes, but the results generally are not so satisfactory, and the characters of the colonies cannot be so readily studied. In this case several agar tubes are taken, a platinum loop is charged with the material to be examined, and in each tube several vertical strokes are made from below upwards on the surface of the agar, one tube after the other being used without recharging the needle. The tubes after inoculation should be kept in the upright position, so that the water of condensation is not allowed to run over the surface.

Dilution Method.—In this method the bacteria are added to the medium when liquid, and mixed by rolling the tube between the palms of the hands (to avoid shaking, which causes air

bubbles); the inoculated medium is then poured out into a capsule (*i.e.* " plated ") and allowed to solidify. As in this case the organisms are diffused throughout the medium, some of the colonies grow on the surface of the medium—" superficial colonies "—others in its substance—" deep colonies." These often show different appearances, which are sometimes used in the systematic description of an organism. As the bacteria may produce too many colonies to allow separation, means must also be used for making different dilutions, a separate plate being

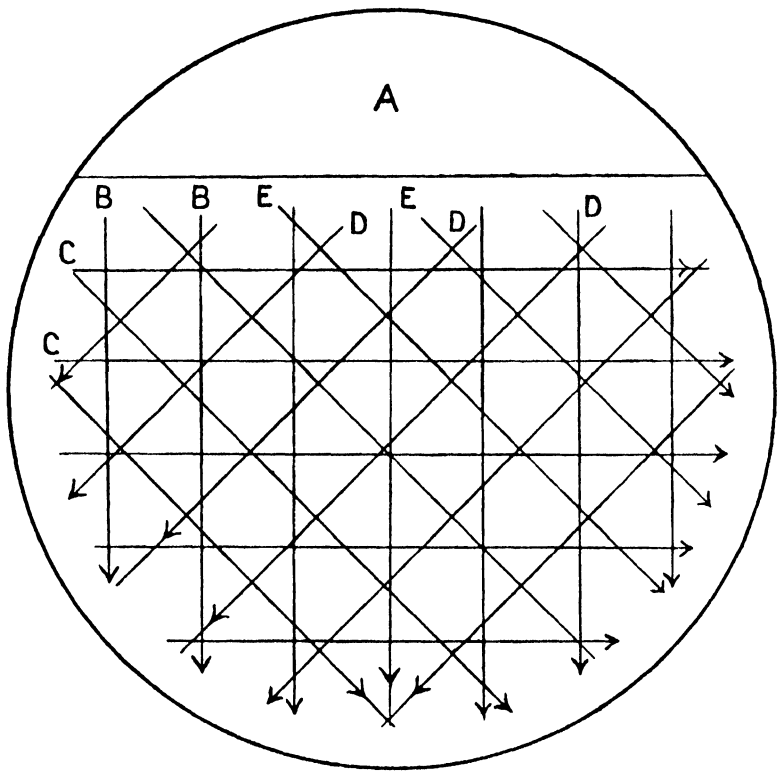


FIG. 20.—Diagram of method of inoculating plate from fæces. (After Medical Research Council's Special Report No. 51.)

prepared for each. If gelatin is used, the medium in tubes is melted and kept in a beaker of water at about 28° C. If agar is used, the medium is melted thoroughly by boiling in a vessel of water and then allowed to cool to about 43° C., at which temperature the inoculations are made. The following are the details :

The contents of three tubes, marked *a*, *b*, *c*,[1] are liquefied as above described. Inoculate *a* with the bacterial mixture. The amount

[1] For marking glass vessels it is convenient to use the red, blue, or yellow oil pencils specially made for the purpose.

of the latter to be taken varies, and can only be regulated by experience. If the microscope shows enormous numbers of different kinds of bacteria present, just as much as adheres to the point of a straight platinum needle is sufficient. If the number of bacilli is small, one to three loops of the mixture may be transferred to the medium. Mix the contents of *a* well. Transfer two loops of medium from *a* to *b*. Mix *b* and transfer five loops to *c* and mix. The plugs of the tubes are in each case replaced and the tubes returned to the beaker. The contents of the three tubes are then poured out into three capsules In doing so the plug of each tube is removed and the mouth of the tube passed two or three times through the Bunsen flame, the tube being meantime rotated round a longitudinal axis Any organisms on its rim are thus killed. The capsules are labelled and set aside till growth takes place.

For accurate work it will be found convenient to carry out the dilutions in definite proportions as follows: In a number of small sterile test-tubes 0·95 c.c. sterile water is put. To the first tube we add 0·05 c c. of the bacterial mixture. The contents of the tube are well shaken up, and the pipette is sterilised by boiling in water. It is allowed to cool, and 0·05 c.c. of fluid is transferred from the first tube to the second. By a similar procedure 0·05 c.c. is transferred from the second to the third, and so on. There is thus effected a twenty-fold dilution in each successive tube. After these steps have been carried out, a definite amount, say 0·05 c.c., is transferred from each tube to a tube of melted medium,—the medium being afterwards plated and the colonies counted when growth occurs. The number of tubes required will vary according to the number of bacteria in the original mixture, but usually four or five will be sufficient.

Enumeration of Colonies.—The dilution method just described supplies the means of counting the number of living bacteria in a fluid, the proviso being always made that they are capable of growth in the medium used. For pathogenic organisms one of the agar media is generally used, whilst in the case of water, gelatin is often employed. The dilutions are made by the quantitative method, and a given amount, say 0·1 c.c., is taken from one of the dilutions and transferred to a tube of melted medium, and, after gentle mixing, the medium is poured in a Petri capsule. It is advisable to take samples in this way from two or even three of the dilutions. To aid the counting of the colonies which develop, various patterns of ruled glass plates have been introduced. If the ruling is in the form of squares of given size, the number of colonies in several squares is counted, and as the area of the Petri dish can be got by multiplying the square of its radius by $3\frac{1}{7}$, the whole number can then be calculated. Petri dishes are rarely flat, and unequal distribution of the colonies has accordingly to be taken into account. The dilution to be selected for taking the sample for plating will depend upon the relative abundance of the organisms in the original fluid.

Separation by Killing Non-spored Forms by Heat.—As has been said, the spores of bacteria resist heat more than the vegetative forms. When a mixture contains spores of one bacterium and vegetative forms of this and other bacteria, then if the mixture be heated for thirty minutes at 80° C. all the vegetative forms may be killed, while the spores will remain alive and will develop subsequently. Several tubes of different media should be inoculated and treated thus, as the success of the method is very variable.

Separation of Pathogenic Bacteria by Inoculation of Animals.—It is found difficult, and often impossible, to separate by ordinary plate methods certain pathogenic organisms, such as B. tuberculosis, B. mallei, and the pneumococcus, when such occur in conjunction with other bacteria. These grow best on special media, and the first two grow so slowly that the other organisms present may outgrow them, cover the whole plates, and make separation difficult. The method adopted in such cases is to inoculate an animal with the mixture of bacilli, wait until the particular disease develops and death occurs, or kill the animal, and with all aseptic precautions (*vide* p. 145) inoculate tubes of suitable media from characteristic lesions situated away from the seat of inoculation.

The Principles of the Culture of Anaerobic Organisms

All ordinary media, after preparation, may contain traces of free oxygen, and will absorb more from the air on standing. (1) For the growth of anaerobes this oxygen may be expelled by the prolonged passing of an inert gas, such as hydrogen, through the medium (liquefied if necessary). Further, the medium must be kept in an atmosphere of the same gas while growth is going on. (2) Media for anaerobes may be kept in contact with the air, if they contain a reducing agent which does not interfere with bacterial growth. Such an agent takes up any oxygen which may already be in the medium, and prevents further absorption. The reducing body used is generally glucose, though formate of sodium may be similarly employed. The preparation of such media has already been described (pp. 49, 50). In this case the medium ought to be of considerable thickness. (3) Anaerobes will grow without excluding the air if the medium contains catalase; this is effected by having portions of fresh animal or vegetable tissue present. But particulate matter of many kinds aids the growth of these organisms.

The Use of Hydrogen for Anaerobic Cultures.—The gas is generated in a large Kipp's apparatus from pure sulphuric acid and pure zinc. It is passed through three wash-bottles, as in Fig. 22. In the first is placed a solution of lead acetate (1 in 10 of water) to remove any traces of sulphuretted hydrogen. In the second is placed a 1 in 10 solution of silver nitrate to remove any arseniuretted hydrogen which may be present if the zinc is not quite pure. In the third is a 10 per cent. solution of pyrogallic acid in caustic potash solution (1 : 10) to remove any traces of oxygen. The tube leading from the last bottle to the vessel containing the medium ought to

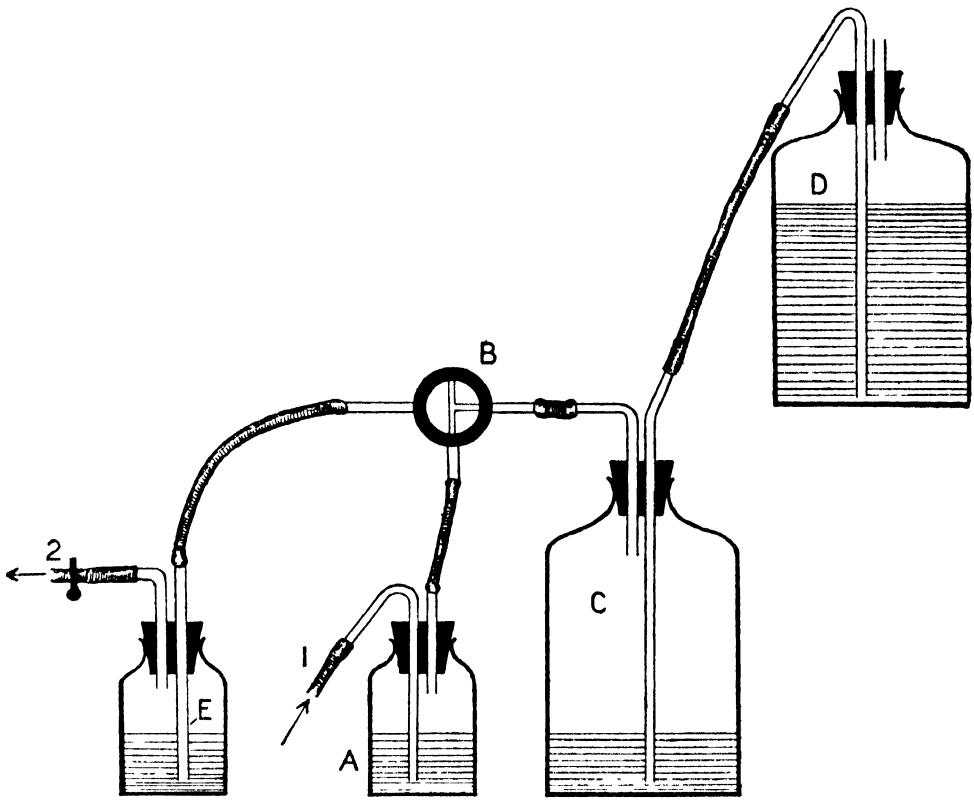


FIG. 21.—Arrangement of bottles for reducing pressure in hydrogen supply from cylinder. (After Mackie and M'Cartney.)

be sterilised by passing through a Bunsen flame, and should have a small plug of cotton wool in it to filter the hydrogen germ-free.

Commercial hydrogen as sold in cylinders may be used; it is necessary, however, to reduce the pressure, and this can be done as follows (Fig. 21). The gas from the cylinder is passed by tube (1), not too rapidly, through the wash-bottle A; from this it passes by the three-way stop-cock B to a jar C of 15–20 litres capacity which is graduated roughly in litres. The jar C is furnished with an indiarubber stopper and tubing as shown. The hydrogen entering C forces the water into a similar jar D which is supported 4 feet above C. When sufficient gas has entered C the supply valve from the cylinder is closed. The stop-cock B is then turned, so that the hydrogen passes from C through the wash-bottle E, and

passes by tube (2) to the anaerobic jar, the flow being adjusted so as to produce a not too rapid stream of bubbles. As the gas escapes from C it is replaced by water from D. All stoppers must be air-tight; this can be secured by means of sealing wax. All the rubber tubing must be of the thick-walled " pressure " type.

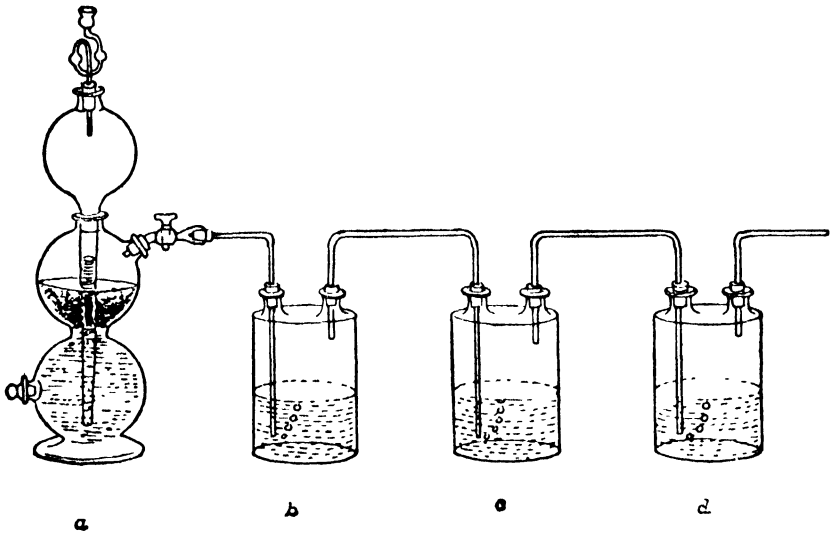


FIG. 22.—Apparatus for supplying hydrogen for anaerobic cultures.

a. Kipp's apparatus for manufacture of hydrogen. *b* Wash-bottle containing 1 : 10 solution of lead acetate *c* Wash-bottle containing 1 : 10 solution of silver nitrate. *d*. Wash-bottle containing 1 : 10 solution of pyrogallic acid. (*b*, *c*, and *d* are intentionally drawn to a larger scale than *a* to show details.)

When it is desired to grow anaerobes on the surface of a solid medium such as agar, tubes of the form shown in Fig. 23, *a* and *b*, may be used. A stroke culture having been made, the air is replaced by hydrogen as just described, and the tubes are fused at the constrictions. Such a method is of great value when it is

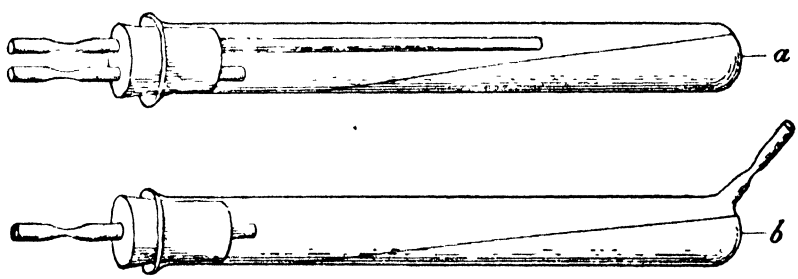


FIG. 23.—Tubes for anaerobic cultures on the surface of solid media.

required to get the bacteria free from admixture of medium, as in the case of staining flagella.

The Use of Pyrogallic Acid and Caustic Potash.—This depends on the fact that pyrogallate of potash rapidly absorbs oxygen from the air (becoming dark brown in colour), thus the air in an enclosed space

can be deprived of its oxygen. Various forms of apparatus have been devised for utilising this property.

Pyrogallate of Potassium for Anaerobic Cultures.—In arranging for the absorption of oxygen by this substance the proportions used in Bulloch's separation method (below) may be employed. Here 109 grams solid caustic potash are dissolved in 145 c.c. water, and to this 2–4 grams pyrogallol are added.

Buchner's Tube is applicable in the case of sloped cultures in test-tubes. This consists of a tube of thick glass measuring about $8\frac{1}{2} \times 1$ inch, with the lower end constricted, so that the culture tube does not reach the foot. The Buchner's tube is provided with a tightly fitting indiarubber stopper. Solid pyrogallic acid (several grams of the heavy " crystals ") is placed in the bottom of the tube; strong caustic potash is added ; then the inoculated culture tube is quickly introduced and the tube is closed with the stopper.

M'Leod's Modification of Lentz's Method.—Shallow circular porcelain capsules [1] (Fig. 24) are used covered in by a porcelain

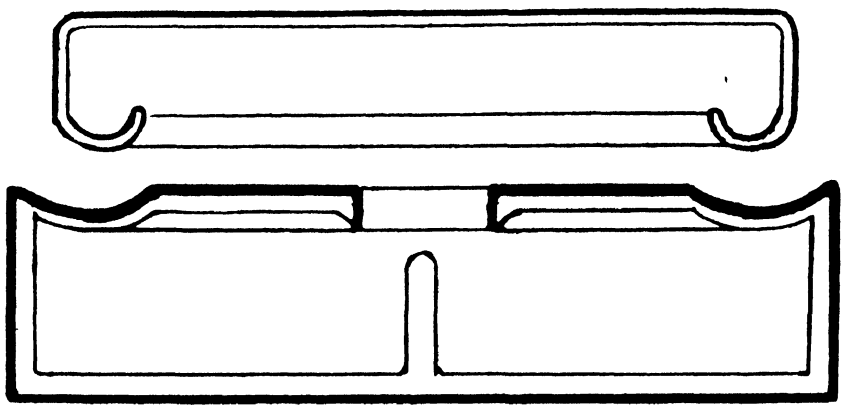


FIG. 24.—M'Leod's capsule for anaerobic plating, shown in section.

diaphragm with the exception of a circular opening in the middle. The interior of each capsule is divided into two halves by a partition, which, however, does not extend the whole way up. In one half, solution of pyrogallic acid is placed; in the other, solution of potassium hydrate. Plasticine is placed round the margin of the upper surface of each capsule. Plate cultures having been made in glass dishes in the usual way, each dish is inverted and placed over a porcelain capsule and carefully fixed in the plasticine. When this has been done, the two fluids in the capsule are mixed by tilting and the oxygen in the interior is rapidly absorbed. Another improvement is that the edges of the glass dishes which rest in the plasticine are turned up so as to prevent the condensation water from running over the plasticine (Fig. 24, *b*).

Henry's Method.—In this modification two shallow circular dishes (portions of Petri capsules) are separated by a tin diaphragm, in the centre of which is an aperture (Fig. 25). The upper dish

[1] The capsules may be obtained from Messrs. Thomson, Skinner & Hamilton, Glasgow.

contains the plate culture, the lower (smaller) contains pyrogallic crystals. Grooves are present in the metal to receive the margins of the dishes, which are fixed in with plasticine. The lower dish is first fixed in position, and just before the upper dish is adjusted,

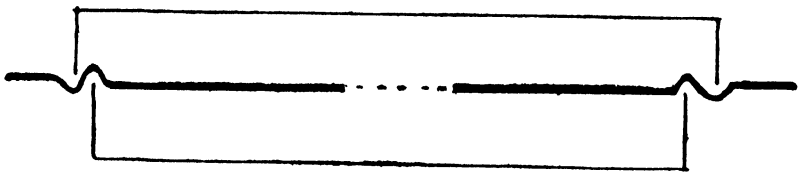


FIG. 25.—Henry's apparatus.

10 c.c. of caustic potash are run into the lower through the opening in the plate.

Bulloch's Apparatus for Anaerobic Culture. — This can be recommended for plating out mixtures containing anaerobes, and for obtaining growths (especially surface growths) of the latter. It consists (Fig. 26) of a glass plate as base on which a bell-jar can be firmly luted down with unguentum resinæ. In the upper part of the bell-jar are two apertures furnished with ground stoppers, and through each of the latter passes a glass tube on which is a stop-cock. One tube, bent slightly just after passing through the stopper, extends nearly to the bottom of the chamber; the other terminates immediately below the stopper. In using the apparatus there is set on the base-plate a shallow dish, of slightly less diameter than that of the bell-jar, and having a little heap of from 2 to 4 grams of dry pyrogallic acid placed in it towards one side. Culture plates, which should be of rather greater thickness than for ordinary aerobic work, can be stacked on a frame of glass rods resting on the edges of the dish, or a beaker containing culture tubes can be placed in it. The bell-jar is then placed in position so that the longer glass tube is situated over that part of the bottom of the shallow dish farthest away from the pyrogallic acid, and the bottom and stoppers are luted. The air in the bell-jar is now expelled by passing a current of hydrogen through the short glass tube, and both stoppers are closed. A partial vacuum is then effected in the jar by connecting up the short tube with an air-pump, opening the tap, and giving a few strokes of the pump. A solution of 109 grams solid caustic potash dissolved in 145 c.c. water is made, and into the vessel containing it a rubber tube connected with the long glass tube is made to dip, and the stopper of the latter being opened, the

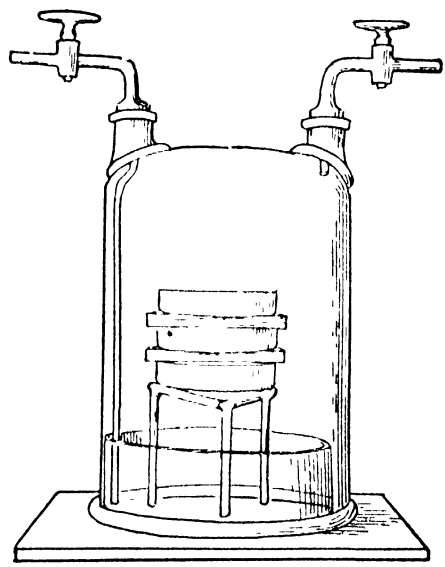


FIG. 26.—Bulloch's apparatus for anaerobic plate cultures.

fluid is forced into the chamber and spreads over the bottom of the shallow dish; potassium pyrogallate is thus formed, which absorbs any free oxygen still present. Before the whole of the fluid is forced in, the rubber tube is placed in a little boiled water, and this, passing through the glass tubes, washes out the potash and prevents erosion of the glass. The whole apparatus may be placed in the incubator till growth occurs.

M'Intosh and Fildes' Anaerobic Jar. — These authors have designed a jar in which tubes may be incubated under anaerobic conditions, the oxygen being absorbed by spongy palladium. A glass jar is employed furnished with a metal lid which can be clamped down. The lid is fitted with a tube and valves so that hydrogen may be admitted into the jar. In the improved form of the apparatus the palladium asbestos is fixed on an insulated spool and surrounded

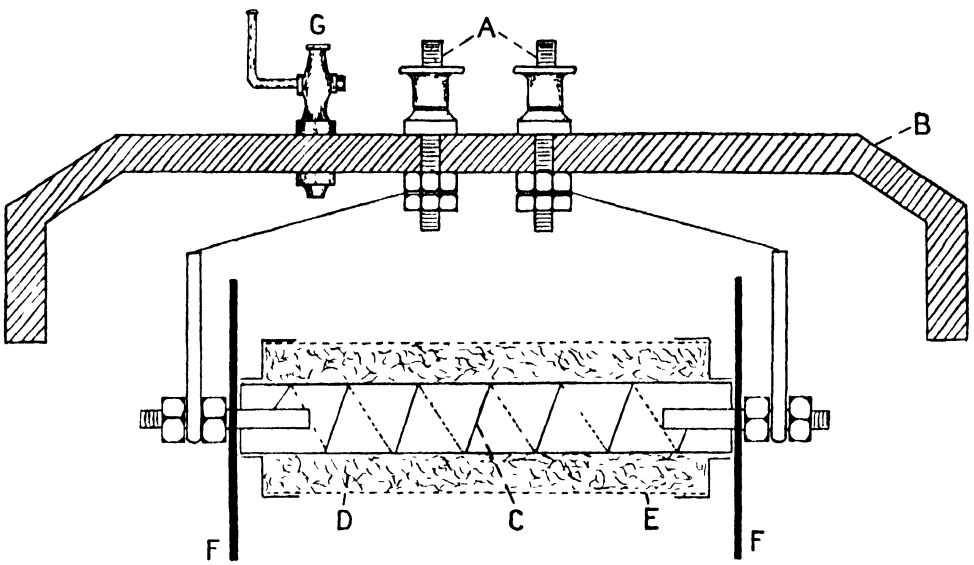


FIG. 27.—Top of anaerobic jar. (Fildes and M'Intosh.)

A Electric terminals B. Section of metal top of jar C. Coil of wire around insulator. D Asbestos. E Gauze cover F Mica plates G. Gas inlet.

by a piece of resistance wire which is connected to two electric terminals on the outside of the lid. Cultures are placed inside the jar and hydrogen is passed through the jar for half a minute. Then the valves are closed and the terminals are attached to the electric supply, which must be reduced by suitable resistances. As a result of the consumption of oxygen by the hot palladium asbestos the pressure within the jar falls, and after the current has been passing for ten minutes more hydrogen may be added at very low pressure; the current should then be allowed to pass for half an hour. Along with the cultures an indicator should be placed in the jar. This consists of a mixture in a test-tube of equal volumes of (*a*) $\frac{N}{10}$ NaOH 6 c.c., water to 100 c.c., (*b*) 3 c.c. $\frac{1}{2}$ per cent watery methylene blue, water to 100 c.c., (*c*) glucose 6 grams, water to 100 c.c., and a small crystal of thymol; this is boiled till reduced and at once placed in the jar. This indicator, when in the jar,

should remain colourless except for a slight tinge of blue at the top, which slowly disappears during the passing of the current. To secure very thorough anaerobiosis, current may be passed at intervals during the period of incubation. It is absolutely necessary that the lid of the jar should be air-tight. This can be tested by placing a few drops of ether in the jar, fixing on the lid, and plunging the vessel in hot water; any leak can thereby be detected.[1]

Another method is to make shake cultures in deep tubes of glucose agar which have been melted and cooled to 45° C. The inoculum is mixed with the agar by rolling the tube, which is then allowed to solidify and is incubated as usual. To make subcultures from separate colonies a capillary pipette (made by drawing out a piece of quill tube) sterilised by heat is passed down to the colony and some of the growth is sucked up into it.

Cultures of Anaerobes.—The obtaining of pure cultures from mixtures of these organisms usually presents very great difficulty, and repeated subculturing from isolated colonies is necessary. When by one or other of the above methods separate pure colonies have been obtained, growth generally may be maintained on media which contain reducing agents, and the test-tubes containing the medium must be filled to a depth of 4 inches. They are sterilised as usual, and are called "deep" tubes (Fig. 14, *c*). The long straight platinum wire is used for inoculating, and it is plunged well down into the "deep" tube. A little air gets into the upper part of the needle track, and no growth takes place there, but in the lower part of the needle track growth occurs. From such "deep" cultures growths may be maintained indefinitely by successive subcultures in similar tubes. Even ordinary gelatin and agar can be used in the same way if the medium is heated to boiling-point before use to expel any absorbed oxygen.

Cultures of Anaerobes in Liquid Media.—Glucose broth is usually most convenient. It is placed either (1) in a conical flask with a lateral opening and a perforated indiarubber stopper, through which a bent glass tube passes (as in Fig. 28, *a*), by which hydrogen may be delivered, or (2) in a conical flask with a rubber stopper furnished with two holes (as in Fig. 28, *b*), through a tube in one of which hydrogen is delivered, while through the tube in the other the gas escapes. The inner end of the gas delivery tube must in either case be below the surface of liquid; the inner end of the lateral nozzle in the one case, and the inner end of the escape tube in the other, must of course be above the surface of the liquid. The single tube in the one case and the two tubes in the other ought to be partially drawn out in a flame to facilitate subsequent com-

[1] A satisfactory form of this apparatus is made by G. E. Burrell, 136 University Avenue, Glasgow.

plete sealing. The ends of the tubes through which the gas is to pass are previously protected by pieces of cotton wool tied on them. It is well also to place in the tube, through which the hydrogen is to be delivered, a little plug of cotton wool. The flask being thus prepared, it is sterilised by methods B (2) or B (3). On cooling it is ready for inoculation. In the case of the flask with the lateral nozzle, the cotton-wool covering having been momentarily removed, a wire charged with the organism is passed down to the bouillon In the other kind of flask the stopper must be removed for an instant to admit the wire. (In the case of many anaerobes it is advisable to make a massive inoculation by adding part of an actively growing bouillon culture) The flask is then connected with the hydrogen apparatus by means of a short piece of sterile india-rubber tubing, and hydrogen is passed through for half an hour. In the case of flask (1), the lateral nozzle is plugged with melted paraffin and covered with alternate layers of cotton wool and paraffin, the whole being tightly bound on with string. The entrance tube is now completely drawn off in the flame before being disconnected from the hydrogen apparatus. In the case of flask (2), first the exit tube and then the entrance tube are sealed off in the flame before the flask is disconnected from the hydrogen apparatus. It is well in the case of both flasks to run some melted paraffin all over the rubber stopper. Sometimes much gas is evolved by anaerobes, and in dealing with an organism where this will occur, provision must be made for its escape. This is conveniently done by leading down the exit tube, and letting the end just dip into a trough of mercury (Fig. 29), or into mercury in a little bottle tied

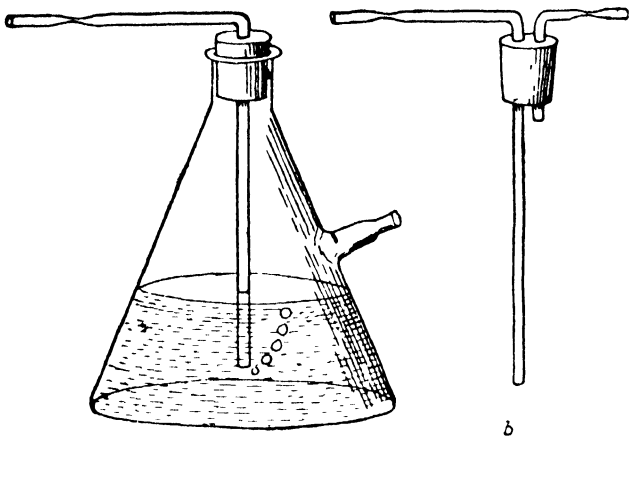


FIG. 28.

a. Flask for anaerobes in liquid media Lateral nozzle and stopper fitted for hydrogen supply.
b. A stopper arranged for a flask without lateral nozzle

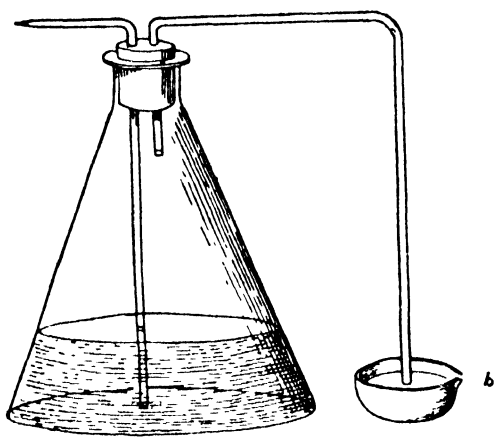


FIG. 29.—Flask arranged for culture of anaerobes which develop gas.

b is a trough of mercury into which exit tube dips

on to the end of the exit tube. The pressure of gas within causes an escape at the mercury contact, which at the same time acts as an efficient valve. This method of culture in fluid media is used to obtain the soluble products of such anaerobes as the tetanus bacillus.

Noguchi's Tubes.—Long narrow tubes ($8 \times \frac{1}{2}$ inches) are half filled with medium and are heated in the Koch for thirty minutes or in boiling water for five minutes. Sterile melted vaselin is then poured on the surface of the medium and the tubes are cooled quickly. Inoculation is effected by means of a capillary pipette after melting the vaselin, which acts as a seal excluding the air. This method is not suitable for the culture of organisms which produce much gas, as the vaselin plug is forced out of the tube.

Method of obtaining growths without exclusion of air (Tarozzi).—If small pieces of fresh sterile organs are added to ordinary bouillon, growth of anaerobes takes place under ordinary atmospheric conditions. For this purpose, portions of liver, spleen, or kidney are most suitable. The method has been used in the cultivation of spirochætes, organism of poliomyelitis, etc. It has been found also that the addition of pieces of boiled vegetable, bran, and even asbestos wool, is effective in making a fluid medium suitable for the growth of anaerobes, the presence of fine interstices in the material being apparently an important factor in aiding the growth (Douglas, Fleming, and Colebrook). Robertson's minced meat medium is very suitable.

The Storing and Incubation of Cultures.—Gelatin cultures must be grown at a temperature below their melting-point, *i.e.* for 10 per cent. gelatin, below 22° C. They are usually kept in ordinary rooms or in a cool incubator at about 20° C. Agar and serum media are employed to grow bacteria at a higher temperature, corresponding to that at which the organisms grow best, usually 37° C. in the case of pathogenic organisms. For the purpose of maintaining a uniform temperature incubators are used. These vary much in the details of their structure, but all consist of a chamber with double walls between which some fluid (water or glycerin and water) is placed. This, when raised to a certain temperature, ensures a fairly constant distribution of the heat round the chamber. The latter is also furnished with double doors, the inner being usually of glass. Heat is supplied from a burner fixed below. These burners vary much in design. Sometimes a mechanism devised in Koch's laboratory is affixed, which automatically turns off the gas if the light be accidentally extinguished. Between the tap supplying the gas, and the burner, is interposed a gas regulator.

The incubators of Hearson of London are fitted with a good regulator (Fig. 30). It is preferable in using an incubator to connect the regulator with the gas supply and with the Bunsen by flexible metal tubing. Electrically heated incubators which do not

require water are very useful. It is necessary to see that there is not too much evaporation from the surface of cultures placed within incubators, otherwise they may quickly dry up It is thus advisable to raise the amount of water vapour in the interior by having in the bottom of the incubator a flat dish full of water from which evaporation may take place. With tubes which will require to be long in the incubator, the plugs should be pushed a little way into the tube and a few drops of melted paraffin dropped on the top of the wool, or the plugs should be covered either by indiarubber caps or by pieces of sheet rubber tied over them These caps should be previously sterilised in 1 : 1000 corrosive sublimate and then dried Before they are placed on the tube the cotton-wool plug ought to be well singed in a flame. Another method of preventing evaporation, which is useful for the maintenance of stock cultures, is to

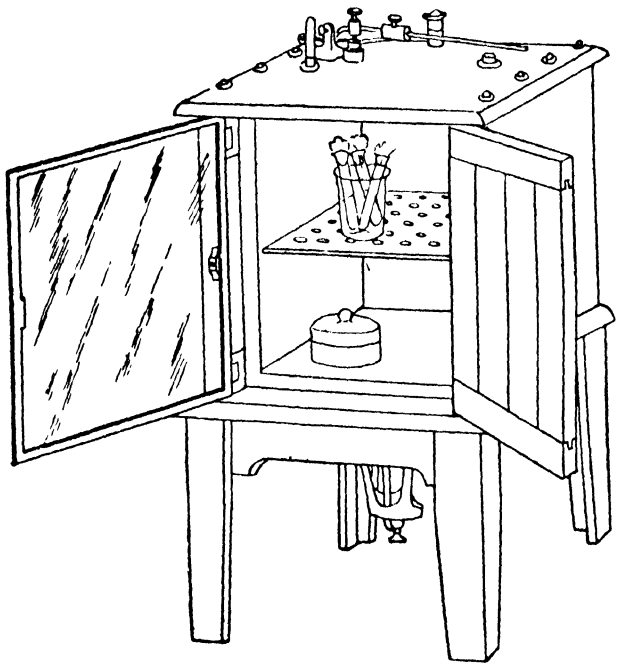


FIG. 30.—Hearson's incubator for use at 37° C. (form heated by gas).

keep them as stab cultures. " Cool " incubators are often used for incubating gelatin at 21° to 22° C. An incubator of this kind fitted with a low-temperature Hearson's regulator is on the market

Filtration of Cultures.—For many purposes it isnecessary to filter all the organisms from fluids in which they may have been growing. This is done especially in obtaining the soluble toxic products of bacteria, and also when investigating " ultramicroscopic " or " filter-passing " viruses. Filters capable of keeping back such minute bodies as bacteria consist of a tube of unglazed earthenware as introduced by Chamberland. The efficiency of such a filter depends on the fineness of the grain of the clay from which it is made ; the finest is the Kitasato filter

and the Chamberland " B " pattern; the next finest is the Chamberland " F " pattern, which is suitable for most purposes. The Doulton porcelain filter is also very efficient. There are several other types of filters, differing slightly in detail, all possessing the common principle, *e.g.* the Berkefeld filter made of diatomaceous earth. In the Seitz filter, a disc of fibrous material is used as the filtering agent; this form has the advantage that the discs are readily replaceable. Extremely fine-pored filters are obtained by the use of collodion membranes. Sometimes the fluid is forced through the filter. Sometimes, when fluids to be filtered are very albuminous, they are forced through a porcelain cylinder by compressed carbonic acid gas. The filtration of albuminous fluids may also be facilitated by keeping them near blood-heat during the process. For ordinary bacteriological work, the most generally convenient filtering apparatus is that in which the fluid is sucked through the porcelain by exhausting the air in the receptacle into which it is to flow. This is conveniently done by means of a Geissler's water-exhaust pump (Fig. 31, *g*), which must be fixed to a tap leading directly from the main. The connection with the tap must be effected by means of a piece of thick-walled rubber-tubing as short as possible, wired on to tap and pump, and firmly lashed externally with many turns of strong tape. Before lashing with the tape the tube may be strengthened by fixing round it with rubber solution strips of the rubbered canvas used for mending punctures in the outer case of a bicycle tyre. A manometer tube (*b*) and a receptacle (*c*) (the latter to catch any back flow of water from the pump which may accidentally occur) are

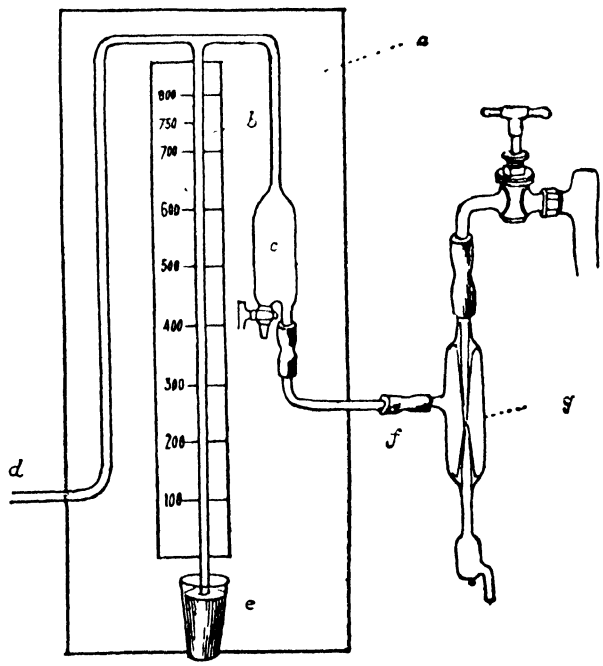


FIG. 31.—Geissler's vacuum pump arranged with manometer for filtering cultures. (The tap and pump are intentionally drawn to a larger scale than the manometer board to show details.)

intercepted between the filter and the pump. These are usually arranged on a board *a*, as in Fig. 31. Between the tube *f* and the pump *g*, and between the tube *d* and the filter, it is convenient to insert lengths of flexible lead-tubing connected up at each end with short, stout-walled rubber-tubing.

Filters are arranged in various ways. (*a*) An apparatus is arranged as in Fig. 32. The fluid to be filtered is placed in the cylindrical vessel *a*. Into this a "candle" or "bougie" of porcelain dips. From the upper end of the bougie a glass tube with thick rubber connections proceeds to flask *b*, and passes through one of the two perforations with which the rubber stopper of the flask is furnished. Through the other

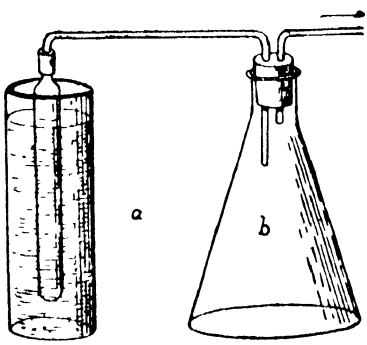


FIG. 32.—Chamberland's candle and flask arranged for filtration.

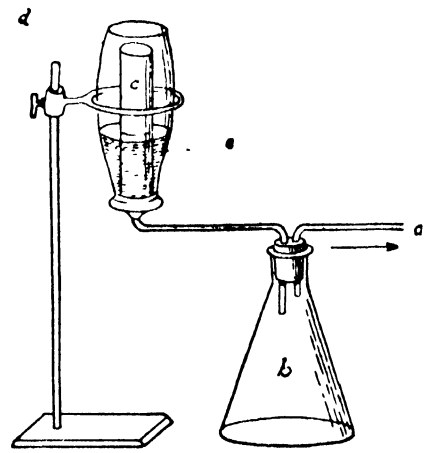


FIG. 33.—Chamberland's bougie arranged with lamp funnel for filtering a small quantity of fluid.

opening a similar tube proceeds to the exhaust-pump. When the latter is put into action the fluid is sucked through the porcelain and passes over into flask *b*. This apparatus is very good, but not suitable for small quantities of fluid.

(*b*) A very good apparatus can be arranged with a lamp funnel and the porcelain bougie. The open end of the filter candle is glazed so as to render it impermeable, or a metal nozzle is cemented into this end (the latter is less suitable). A tightly fitting perforated indiarubber bung is slipped over this part of the filter; the lower part of the bung fits into the neck of the flask, the upper part fits into a glass cylinder (Figs. 33, 34). The efficiency of such a filter, especially when small amounts of fluid are being dealt with, is much increased if when the level of the fluid falls below the upper end of the candle a closely

fitting test-tube is slipped over the latter. By this device the leakage of air through the exposed part of the candle is prevented.

(*c*) Muencke's modification of the Chamberland filter is seen in Fig. 35. It consists of a thick-walled flask *a*, the lower part conical, the upper cylindrical, with a strong flange on the lip. There are two lateral tubes, one horizontal to connect with exhaust-pipe, and one sloping, by which the contents may be poured out. Passing into the upper cylindrical part of the flask is a hollow porcelain cylinder *b*, of less diameter than the cylindrical part of flask *a*. It is closed below, open above, and rests by a projecting rim on the flange of the flask, an asbestos washer, *c*, being interposed, or it may slip into a perforated indiarubber bung which fits the neck of the flask. The fluid to be filtered is placed in the porcelain cylinder, and the whole top covered, as shown at *f*, with an indiarubber cap with a central perforation (the latter, however, is unnecessary where a bung is used); the tube *d* is connected with the exhaust-pump, and the tube *e* plugged with a rubber stopper.

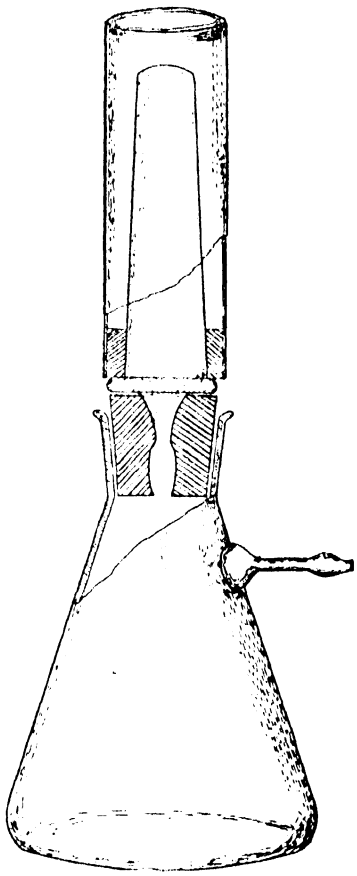


FIG. 34.—Another form of arrangement of filtering candle and flask.

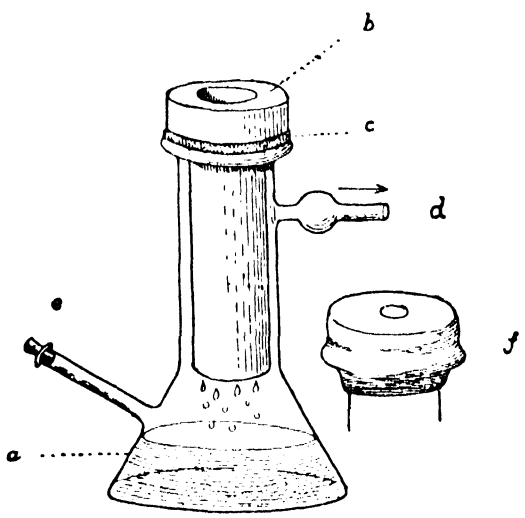


FIG. 35.—Muencke's modification of Chamberland's filter.

Before any one of the above apparatus is used it ought to be connected up as far as possible and sterilised in the Koch's steriliser. The ends of any important unconnected parts ought to have pieces of cotton wool tied over them. After use the bougie is to be sterilised

in the autoclave. Much of the material kept back on the filter can now be removed by forcing water through in a direction opposite to that of the flow of the fluid during filtration. Alternatively, the candle, after being dried, should be passed carefully through a Bunsen flame to burn off all organic matter. If the latter is allowed to accumulate, the pores become filled up. It is to be noted, however, that the efficiency of a filter does not depend solely on the size of its pores; these points are discussed further when speaking of ultra-microscopic viruses (p. 611).

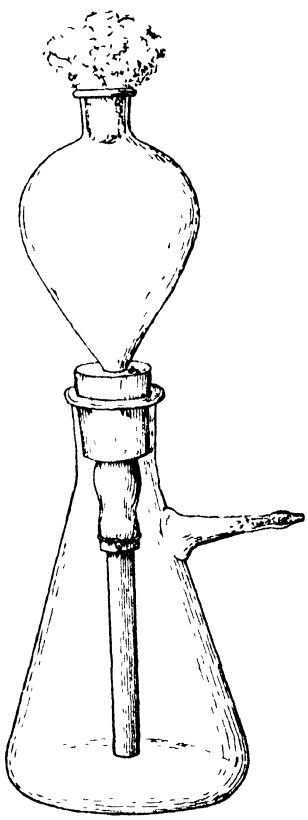


FIG. 36. — Flask for filtering small quantities of fluid.

The success of filtration must be tested by inoculating tubes of media from the filtrate, and observing if growth takes place, as there may be minute perforations in a candle sufficiently large to allow bacteria to pass through. It is often advisable to add to the fluid immediately before filtration a suspension of an agar culture of an easily identifiable organism such as Staphylococcus aureus or B. prodigiosus. Filtered fluids keep for a long time if the openings of the glass vessels in which they are placed are kept thoroughly closed, and if these vessels be stored in a cool place in the dark. A layer of sterile toluol about half an inch thick ought to be run on to the top of the filtered fluid to protect it from the atmospheric oxygen.

Instead of being filtered off, the bacteria may be killed by various antiseptics, chiefly volatile oils, such as oil of mustard (Roux). These oils are stated to have no injurious effect on the chemical substances in the fluid, and they may be subsequently removed by evaporation. It is not practicable to kill the bacteria by heat when their soluble products are to be studied, as many of the latter are destroyed by a lower temperature than is required to kill the bacteria themselves.

Bacteria can be almost entirely removed from fluid cultures by spinning the latter in a centrifuge of very high speed (*e.g.* C. J. Martin's turbine centrifuge), and this method is sometimes adopted in practice.

Centifruges.—For many purposes of bacteriological investigation a centrifuge is essential. Various forms are available, but the apparatus should be capable of holding four tubes each of 15–30 c.c. capacity. For ordinary purposes a speed up to 4000 revolutions per minute should be obtainable ; the motive power may be water or electricity, but hand-driven centrifuges may be used.

The Drying of Substances *in vacuo*.—As many substances, for example toxins and antitoxins, with which bacteriology is concerned would be destroyed by drying with heat as is done in ordinary chemical work, it is necessary to remove the water at

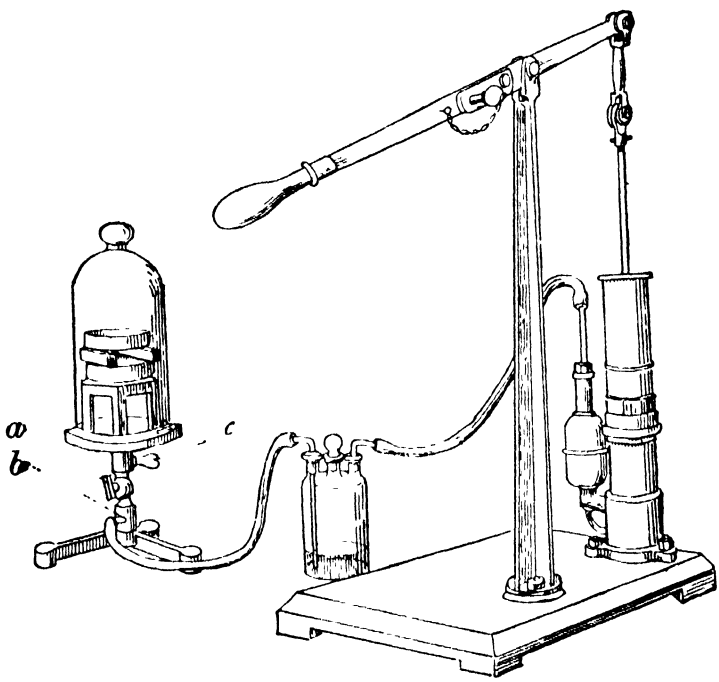


FIG. 37.—Geryk air-pump for drying *in vacuo*.

the ordinary room temperature. This is most quickly effected by drying *in vacuo* in the presence of some substance, such as strong sulphuric acid, which readily takes up water vapour. The vacuum produced by a water-pump is here not available, as in such a vacuum there must always be water vapour present. An air-pump is therefore to be employed. Here we have found the Geryk pump very efficient, and it has this further advantage, that its internal parts are lubricated with an oil of very low vapour density, so that almost a perfect vacuum is obtainable.

The apparatus is shown in Fig. 37. The vacuum chamber consists of a bell-jar set on a brass plate. A perforation in the centre

of the latter leads into the pipe *b*, which can be connected by strong-walled rubber-tubing with the air-pump, and which can be cut off from the latter by a stop-cock *a*. In using the apparatus the substance to be dried is poured out in flat dishes (one-half of a Petri capsule does very well), and these are stacked alternately with similar dishes of strong sulphuric acid on a stand which rests on the brass plate. The edge of the bell-jar is well luted with unguentum resinæ and placed in position and the chamber exhausted. In a few hours, if, as is always advisable, each dish have contained only a thin layer of fluid, the drying will be complete. The vacuum is then broken by admitting air very slowly through a bye-pass *c*, and the bell-jar is removed. In such an apparatus it is always advisable, as is shown in the figure, to have interposed between the pump and the vacuum chamber a Wolff's bottle containing sulphuric acid. This protects the oil of the pump from contamination with water vapour. Whenever the vacuum is produced the rubber-tube should be at once disconnected from *b*, the cock *a* being shut. It is advisable when the apparatus is exhausted to cover the vacuum chamber and the Wolff's bottle with wire guards covered with strong cloth, in case, under the external pressure, the glass vessels give way. The connecting and disconnecting of rubber-tubing of sufficient thickness to withstand collapse when exhausted is difficult. Ordinary stout rubber-tubing can be used if through it there is passed a length of narrow flexible metal-tubing, the ends of which project beyond the rubber-tubing so as to enter the parts of the apparatus to which the latter is fitted. For special purposes other methods are available.

Method of Mounting Bacterial Cultures as Permanent Museum Specimens (Richard Muir).—(*a*) *Stab or Stroke Cultures in Nutrient Gelatin or Agar Media.*—When the culture shows typical characters, further growth is arrested by placing the tube in a formol vapour chamber, or by saturating the cotton-wool plug with strong formalin. Then leave for a day or two. Make up the following :

(1)	Thymol water (saturated in cold) . .	100 c.c.
	Glycerin	20 ,,
	Acetate of potash	5 grams
	Coignet's (gold label) gelatin . . .	10 ,,

Render the mixture acid to litmus with acetic acid ; clear with white of egg, and filter.

Warm to about 40° C , and, removing cotton-wool plug from culture, take a little of the preserving fluid in a pipette and allow to run gently over surface of medium in tube. Place in such a position that a thin layer of the preserving medium remains completely covering the growth and the surface of culture medium. The gelatin is now allowed to solidify. Add three or four drops of strong formalin to the tube, and fill up to within a quarter of an inch of the top of the tube with the following fluid :

(2)	Thymol water (saturated in cold) . .	100 c.c.
	Glycerin	20 ,,
	Acetate of potash	5 grams.

Cover top of tube with a small piece of paper so as to keep out

dust, allow to stand for a day or two so that small air-bells may rise to the surface.

To seal tube, pour melted paraffin gently on to the surface of fluid up to near the top of tube ; allow to solidify. Cover paraffin with layer of alcoholic orange shellac cement ; allow this to set, and repeat until the cement becomes level with top of test-tube. When the cement is set, a few drops of black lacquer are put on, and a circular cover-glass of about the same diameter as the mouth of tube is placed so as completely to seal it.

(*b*) The following method is useful for preserving *plate cultures* : Instead of making the cultures in Petri's capsules, use ordinary watch-glasses The watch-glass is sterilised in a Petri's capsule, and the inoculated medium is poured out into the watch-glass, allowed to solidify in the usual way, and left in the Petri's capsule until the colonies of growth have developed The watch-glass is now removed from capsule, and a layer of the preserving gelatin medium (1) (p. 92), to which have been added a few drops of strong formalin, is allowed to spread over the surface of the culture medium When the layer is solidified the watch-glass is filled up with the same, and a clean square or oblong piece of glass (which, of course, should be of slightly larger diameter than the watch-glass) is now carefully placed over watch-glass, care being taken that no air-bells are formed. The edge of watch-glass should be closely applied to the glass cover, and left in position until the gelatin has solidified. The superfluous gelatin is now removed, and the glasses sealed first with the orange shellac cement, then with black lacquer. It is now finished off by using a circular mask of suitable size

The various kinds of solid media used in the cultivation of bacteria, such as blood serum, potato, bread paste, etc., can be treated in the same manner with excellent results.

General Laboratory Rules.—On the working bench of every bacteriologist there should be a large dish of 1 : 1000 solution of mercuric chloride in water, or 10 per cent. liquor cresolis saponatus. Into this all tubes, vessels, plates, hanging-drop cultures, etc., which have contained non-sporing bacteria and with which he has finished, ought to be at once plunged (in the case of tubes, the tube and plug should be put in separately). On no account whatever are such infected articles to be left lying about the laboratory. The basin is to be repeatedly cleaned out. All the glass is carefully washed in repeated changes of tap water to remove the last trace of perchloride of mercury, a very minute quantity of which is sufficient to inhibit growth. Old cultures which have been stored for a time, and from which fresh sub-cultures have been made, ought to be steamed in the Koch's steriliser for two or three hours, or in the autoclave for a shorter period, and the tubes thoroughly washed out. Besides a basin of antiseptic solution for infected apparatus, etc., there ought to be a second reserved for the worker's hands in case of any accidental contamination. When a large number of tubes are

being daily put out of use, they may be placed in an enamelled iron pail, and this when full is placed in the steam steriliser. Cultures and other materials containing sporing organisms, *e.g.* B. anthracis or B. tetani, ought to be autoclaved.

A white glazed tile on which a bell-jar can be set is very convenient to have on a bench. Infective material in watch-glasses can be placed thus under cover, and if anything is spilled the whole can be easily disinfected. In making examinations of organs containing virulent bacteria, the hands should be previously dipped in 1 : 1000 mercuric chloride and allowed to remain wet with this solution. No food ought to be partaken of in the laboratory, and pipes, etc., are not to be laid with their mouth-pieces on the bench. No label is to be licked with the tongue. Before leaving the laboratory the bacteriologist ought to wash the hands and forearms with antiseptic solution and then with yellow soap. In the case of any fluid containing bacteria being accidentally spilt on the bench, etc., 10 per cent. liq. cresol. sap. is to be at once poured on the spot. The air of the laboratory ought to be kept as quiet as possible.

CHAPTER III

MICROSCOPIC METHODS

The Microscope—For ordinary bacteriological work a good microscope is essential. It ought to have a heavy stand, with coarse and fine adjustments, a double mirror (flat on one side, concave on the other), a good condenser, with an iris diaphragm, and a triple nose-piece. Three objectives are necessary, ordinary low and high powers ($\frac{2}{3}$ and $\frac{1}{6}$ inch) and a $\frac{1}{12}$-inch oil immersion. It is well to have two eyepieces. The flat side of the mirror ought to be used along with the condenser. It may here be remarked that when it is desired to bring out in sharp relief the margins of unstained objects, *e.g.* living bacteria in a fluid, a narrow aperture of the diaphragm should be used ; whereas, in the case of stained bacteria, when a pure coloured picture is desired, as in examining stained films, the diaphragm ought to be *widely opened.* The condenser should be at such a height that the rays of light from the illuminant are brought to a focus in the plane of the preparation to be examined ; as a rule, this result is attained when the upper surface of the condenser is practically on the same level as the stage of the microscope. To adjust the condenser accurately, place a microscopic preparation on the stage and focus it with the low-power objective. Then, keeping the position of objective unchanged, rack the condenser till an image of the lamp is seen sharply ; if daylight is used, focus a part of the window frame. The condenser is then at the proper level for use with the immersion lens. In *examining* specimens with the low-power objective, lower the condenser till an equally lit field is obtained.

The oil immersion lens is used as follows. After the light is satisfactorily arranged, fix the slide on the stage with the right-hand clip and place a drop of cedar-wood oil on the preparation ; lower the objective till the point touches the drop of oil. Then with the eye at the ocular, rack the tube of the microscope slowly down till the preparation comes into focus ; then focus with the fine adjustment. If the microscope is not fitted with a movable stage, it is convenient to use the left hand for

moving the preparation whilst the right operates the fine adjustment. When the observer has finished for the time being with the immersion lens he ought to wipe off the oil with a piece of silk or very fine washed linen. If the oil has dried on the lens it may be moistened with xylol—never with alcohol, which will dissolve the material by which the lens is fixed in its metal carrier.

Microscopic Examination of Bacteria.—1. Hanging-drop Preparations.—Micro-organisms may be examined: (1) alive or dead in fluids; (2) in film preparations; (3) in sections of tissues. In the two last cases advantage is always taken of the affinity of bacteria for certain stains. When they are to be examined in fluids a drop of the liquid may be placed on a slide and covered with a cover-glass.[1] It is more usual, however, to employ hanging-drop preparations. The technique of making these has already been described (p. 70). In examining them microscopically, it is necessary to use a small diaphragm. It is best to focus the edge of the drop with a low-power objective, and, the slide being arranged so that part of the edge crosses the centre of the field, to clamp the preparation in this position. A high-power lens is then turned into position, and lowered by the coarse adjustment to a short distance above its focal distance; it is now carefully screwed down by the fine adjustment, the eye being kept at the tube meanwhile. The shadow of the edge will be first recognised, and then the bacteria must be carefully looked for. Often a dry lens is sufficient, but for some purposes the oil immersion is required. If the bacteria are small and motile, a beginner may have great difficulty in seeing them, and it is well to practise at first on some large non-motile form, such as anthrax. In fluid preparations the natural appearance of bacteria may be studied, and their rate of growth determined. The great use of such preparations, however, is to find whether or not the bacteria are motile, and for determining this point it is advisable to use either broth or agar cultures not more than twenty-four hours old, or preferably younger. In the case of solid cultures, a small fragment of growth is broken down in broth or in sterile water. In determining whether or not a bacterium is motile, great difficulty is often experienced in distinguishing between true motion and Brownian movement, especially if the organism be small. The essential criterion to be fulfilled is that the bacteria shall be moving in all directions,

[1] In bacteriological work it is essential that cover-glasses of No. 1 thickness (*i.e.* 0·14 mm. thick) should be used, as those of greater thickness are not suitable for a $\frac{1}{12}$-inch lens.

the observation of individuals close together starting to move in opposite directions being important. The observation of hanging-drop preparations must be correlated with the results of staining for the presence of flagella which, so far as is known, are present in all ordinary motile forms.

Dark-Ground Illumination.—The principle of this method is that the object to be examined is illuminated by rays of light brought to a focus on the object from all sides, no direct rays

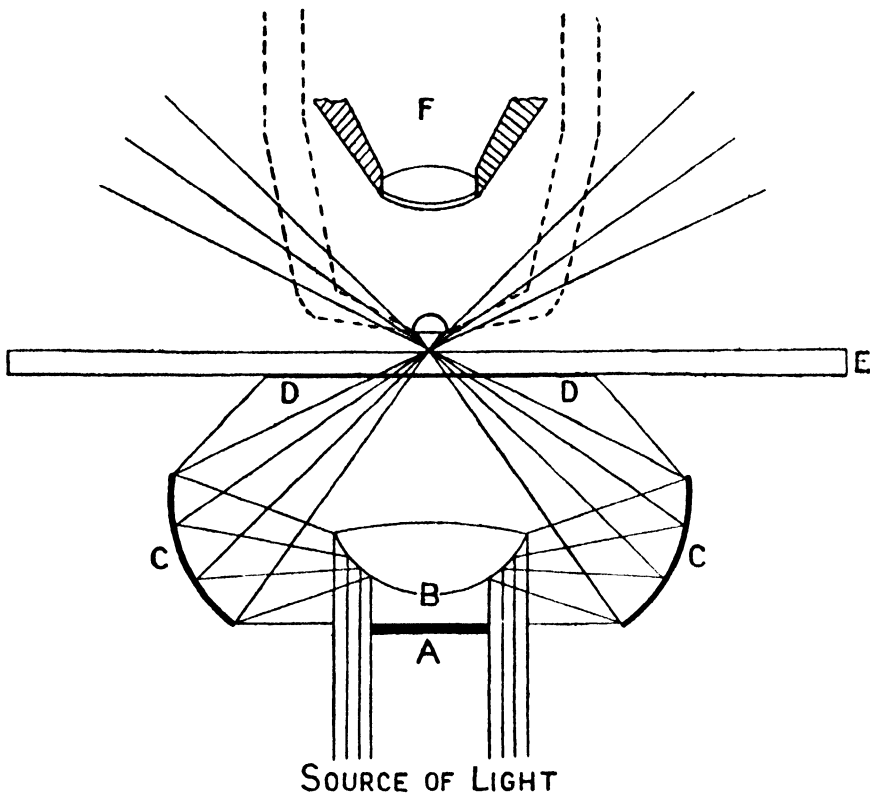


FIG. 38.—Diagram showing course of rays in dark-ground illumination.

A. Circular stop. B and C Reflecting surfaces D Drop of oil between condenser and slide E Microscopic slide F Lens

from the source of illumination reaching the eye of the observer. The object is thus seen brightly lighted on a dark background. A special form of condenser is required, which may be of the spherical or paraboloid type. In the former, the central rays of the beam of light are interrupted by a circular diaphragm while the lateral rays are reflected outwards from the lower surface of a spherical reflector and then inwards from another reflecting surface, and are brought to a focus in the position of the object to be examined (Fig. 38). Within the oil immersion

lens to be used a stop is placed so as to cut down its N.A. to 1 or less. The source of light is usually a small arc lamp or a "pointolite," with a condensing lens which forms a beam of approximately parallel rays. The following are the steps in the procedure :

(1) The microscope with special condenser is placed in front of the source of light so that the beam of light occupies the surface of the substage mirror, the flat surface being used and the mirror being placed at an angle to direct the vertical rays of light upwards in the optical axis (2) Centre the illumination. To do this use a low-power ocular ; remove the condenser and swing out the objective. Then, holding a card or sheet of paper above the ocular, at right angles to the axis of the microscope tube, adjust the mirror so that a uniform disc of light is seen on the paper. (3) Replace the condenser and rack it up to the level of the stage. Place a drop of oil free from bubbles on the upper surface of the condenser ; then rack the latter down a little Place the preparation to be examined on the stage and fix it with the clips. Then rack the condenser slowly up till the oil spreads in a uniform layer between the upper surface of the condenser and the slide (Fig. 38) (5) Focus the preparation with a low-power objective Rack the condenser a little till a bright spot of light is seen and then bring this by means of the adjusting screws of the condenser into the middle of the field. (6) Put a drop of oil on the cover-glass of the preparation Place the immersion lens (fitted with the stop as described) in position and focus in the usual way A little adjustment of the mirror or of the centring screws may be necessary to get the optimum result - brilliantly lighted objects on a black background.

It is a great advantage to use a separate microscope for dark-ground work and to have it mounted on a wooden stand along with the electric lamp Zeiss supplies a very convenient form of condenser in which rapid change may be made from dark-ground to ordinary direct illumination.

NOTE —Slides to be used for the dark-ground method ought to be not more than 1·2 mm. in thickness ; if thicker, the light will be brought to a focus below the level of the film

2. **Film Preparations.**—(*A*) *Dry Method.*—This is the most extensively applicable method for microscopically examining bacteria. For all ordinary purposes films are made on slides, but sometimes when several manipulations have to be performed, as in the staining of flagella, cover-glasses will be found more convenient. Fluids containing bacteria, such as blood, pus, scrapings of organs, can be thus investigated, as also cultures in fluid and solid media. The first requisite is a perfectly clean slide or cover-glass. Many methods are recommended for obtaining such. The test of this being accomplished is that, when the drop of fluid containing the bacteria is placed upon the glass, it can be uniformly spread with the platinum needle all over the surface without showing any tendency to retract into

droplets. The best method is that recommended by van Ermengem. The cover-glasses or slides are placed for some time in a mixture of concentrated sulphuric acid 6 parts, potassium bichromate 6 parts, water 100 parts. After thorough washing in water they may be kept in 50 per cent. alcohol ; for use, they are dried with a soft clean cloth. If a fluid is to be examined, a loopful may be placed on the cover-glass and spread out over the surface with the needle. When a culture on a solid medium is to be examined, a loopful of water is placed on the slide, and a minute particle of growth rubbed up in it and spread over the glass, so as to form a thin film. The great mistake made by beginners is to take too much of the growth. The point of the straight needle should just touch the surface of the culture, and when this is rubbed up in the droplet of water and the film dried, there should be an opaque cloud just visible on the slide. In the case of *pus* or *sputum* a loopful should be spread out on the slide so as to make streaks of varying thickness. When a film has been spread, it must next be dried by being waved backwards and forwards at arm's-length above a Bunsen flame. The film must then be fixed on the glass by being passed three or four times slowly through the flame. In doing this a good plan is to hold the glass between the right forefinger and thumb ; if the fingers just escape being burned no harm will accrue to the bacteria in the film. When cover-glasses are used for films, Cornet's forceps will be found very convenient for handling them (Fig. 39).

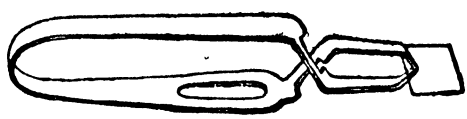


FIG. 39.—Cornet's forceps for holding cover-glasses.

In the case of *blood*, a small drop is placed near one end of a clean slide, the edge of a second slide is lowered through the drop on to the surface of the glass on which the blood has been placed. This second slide is held at an angle to the first, and the droplet of blood by capillarity spreads itself in the angle between the two slides. The edge of the second slide is then stroked along the surface of the first slide, and the blood is spread out in a film whose thickness can be regulated by the angle formed by the second slide. Large-sized films can thus be obtained. A film prepared in this way may be too thick at one edge, but at the other is beautifully thin. Another method is to allow a drop of blood to spread itself between two clean cover-glasses, which are then to be slipped apart, and being held between the forefinger and thumb are to be dried by a

rapid to-and-fro movement in the air. If it is desired to preserve the red blood corpsucles in a film, it may be fixed by one of the following methods: by being placed (*a*) in a hot-air chamber at 120° C. for half an hour; (*b*) in methyl alcohol for two to three minutes, then washed and dried; (*c*) in formol-alcohol (Gulland)—formalin 1 part, absolute alcohol 9 parts—for five minutes, then washed and dried. In using the Romanowsky stains no previous fixation is necessary (*vide infra*).

In the case of *urine*, the specimen must be allowed to stand, and films made from any deposit which occurs; or, what is still better, the urine is centrifuged and films are made from the deposit. After dried films are thus made from urine it is an advantage to place a drop of distilled water on the film and heat gently to dissolve the deposit of salts; then gently wash in water and dry. In this way a much clearer picture is obtained when the preparation is stained.

Films dried and fixed by the above methods are now ready to be stained by the methods to be described below.

(*B*) *Wet Method.*—If it is desired to examine the fine histological structure of the cells of a discharge as well as to investigate the bacteria present, it is advisable to substitute "wet" films for the "dried" films, the preparation of which has been described. The nuclear structure, mitotic figures, etc., are by this method well preserved, whereas these are considerably distorted in dried films. The initial stages in the preparation of wet films are the same as above, but instead of being dried in air they are placed, while still wet, film downwards in the fixative. The following are some of the best fixing fluids:

(*a*) Zenker's solution (p. 119) or a saturated solution of perchloride of mercury in 0·75 per cent. sodium chloride; fix for five minutes. Then place the films for half an hour, with occasional gentle shaking, in 0·75 per cent. sodium chloride solution to wash out the corrosive sublimate; they are thereafter washed in successive strengths of methylated spirit, treated with Gram's iodine to remove excess of mercuric salt, and again with spirit After this treatment the films are stained and treated as if they were sections.

(*b*) Formol-alcohol—formalin 1 part, absolute alcohol 9. Fix films for three minutes; then wash well in methylated spirit. They are then stained, etc, as in (*a*). This is an excellent and very rapid method

(*c*) Corrosive-alcohol—alcohol 1 part, saturated solution of corrosive sublimate 2 parts. Fix for five minutes, wash with 50 per cent. spirit, and treat as in (*a*). This fixature is very suitable for films of fæces containing entamœbæ (p. 674).

3. **Examination of Bacteria in Sections.**—The methods of fixing and cutting tissues will be described below. Paraffin sections give by far the best results, and we shall suppose that the sections have been fixed on slides by the method given (p. 121).

The procedure is then as follows: A drop or two of xylol is placed on the section and made to move backwards and forwards; it is poured off and the process is repeated. The excess of xylol is then removed by a clean cloth, care being taken not to allow the preparation to dry. A few drops of absolute alcohol are made to run through the preparation so as to remove the xylol. This is followed in the same way by methylated spirit, and the slide is then plunged in a bowl of water. In the following account we shall assume that the sections have all been brought to this stage. Sections cut on the freezing microtome are also brought into water and are then to be similarly treated.

Sections after being stained are (*a*) dehydrated, (*b*) cleared, and (*c*) mounted in Canada balsam. Dehydration is usually effected by alcohol. The preparation is blotted with filter paper and treated with a few drops of alcohol, then cleared in xylol, and mounted in a drop of balsam. After being treated with xylol the preparation ought to be perfectly clear; if any opacity remains, further dehydration with alcohol is necessary. For certain purposes, *e.g.* when the bacteria are readily decolorised by alcohol, aniline-xylol (aniline oil 2 parts, xylol 1 part) is used as the dehydrating agent. The preparation is blotted as before and treated with aniline-xylol which clears at the same time. The aniline-xylol is then replaced by xylol; this is conveniently done by running the xylol through the preparation in one direction, when the aniline-xylol will be seen to be displaced before it. The preparation is then mounted in balsam.

The Staining of Bacteria

Staining Principles.—To speak generally, the protoplasm of bacteria reacts to stains in a manner similar to the nuclear chromatin, though sometimes more and sometimes less actively. The bacterial stains *par excellence* are the basic aniline dyes. These dyes are more or less complicated compounds derived from the coal-tar product aniline. Many of them have the constitution of salts. Such compounds are divided into two groups according as the staining action depends on the basic or the acid portion of the molecule. Thus the acetate of rosaniline derives its staining action from the rosaniline. It is therefore called a basic aniline dye. On the other hand, ammonium picrate owes its action to the picric acid part of the molecule. It is therefore termed an acid aniline dye. These two groups have affinities for different parts of the animal cell. The basic stains have a special affinity for the nuclear chromatin, the acid

for the cytoplasm and various formed elements. Thus it is that the former—the basic aniline dyes—are especially the bacterial stains.

The number of basic aniline stains is very large. The following are the most commonly used :

Violet Stains.—Crystal violet and the closely-related methyl-violet and gentian-violet.

Blue Stains.—Methylene-blue [1] (synonym : phenylene-blue).
Victoria-blue.
Thionin-blue.

Red Stains.—Basic fuchsin (synonyms : basic rubin, magenta).
Safranin (synonyms : fuchsia, giroflé).

Brown Stain.—Bismarck-brown (synonyms : vesuvin, phenylene-brown).

Of the stains specified, the violets and reds are the most intense in action, especially the former. It is thus easy in using them to overstain a specimen. Of the blues, methylene-blue probably gives the best differentiation of structure, and it is difficult to overstain with it. Thionin-blue gives also good differentiation and does not readily overstain. Its tone is deeper than that of methylene-blue, and it approaches the violets in tint.

It is most convenient to keep saturated alcoholic solutions of the stains made up, and for use to dilute a little with ten times its bulk of distilled water and filter. A solution of good body is thus obtained. Most bacteria (except those of tubercle, leprosy, and a few others) will stain in a short time in such a fluid. Watery solutions may also be made up, *e.g.* a saturated watery solution of methylene-blue or a 1 per cent. solution of gentian-violet. Stains must always be filtered before use ; otherwise there may be deposited on the preparation granules which it is impossible to wash off. The violet stains in solution in water have a great tendency to decompose. Only small quantities should therefore be prepared at a time.

The Staining of Films.—Films are made on slides from *cultures* as described above, and a few drops of the stain are placed on the surface. When the preparation has been exposed for the requisite time, usually a few minutes, it is well washed in tap water in a bowl, or with distilled water with such a simple siphon arrangement as that figured (Fig. 40). The figure explains itself. When the film has been washed the surplus of water is drawn off with a piece of filter paper, the preparation is blotted and carefully dried high over a flame, a drop of cedar-

[1] This is to be distinguished from methyl-blue, which is a different compound.

wood oil is placed on the film and the preparation is examined. If the preparation is to be preserved, the cedar-wood oil should be removed by xylol which is then dried off. Such a preparation, if kept free from dust, may be preserved for a long time in the unmounted state; or it may be mounted in neutral Canada balsam with a cover-glass. In the case of films on cover-glasses, the preparations are dried as above described and then mounted in balsam.

Films of *fluids from the body* (blood, pus, etc.) can be generally stained in the same way, and this is often quite sufficient for diagnostic purposes. The blue dyes are here preferable, as they do not readily overstain. In the case of such fluids, if the histological elements also claim attention it is best first to stain the cellular protoplasm with 1 to 2 per cent. watery solution of eosin (which is an acid dye), and then to use a blue which will stain the bacteria and the nuclei of the cells. The Romanowsky stains (*vide* p. 116) are here most useful, as by these the preparations are fixed as well as stained.

For the general staining of films a saturated watery solution of methylene-blue will be found to be the best stain to commence with; the Gram method (*vide infra*) is also used, and subsequently any special stains which may appear advisable.

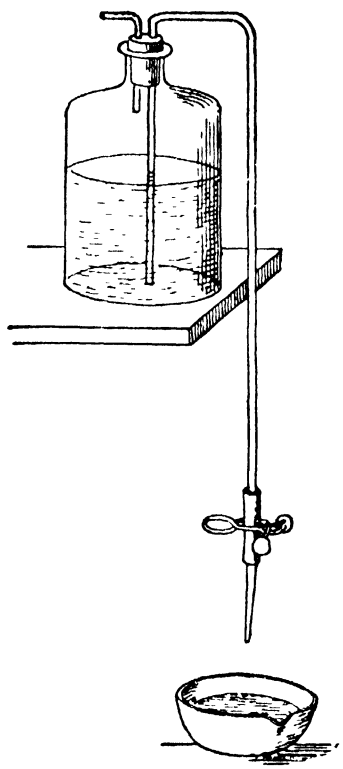


FIG. 40. — Siphon wash-bottle for distilled water used in washing preparations.

The Use of Mordants and Decolorising Agents.—In films of blood and pus, and still more so in sections of tissues, if the above methods are used, the tissue elements may be stained to such an extent as quite to obscure the bacteria. Hence many methods have been devised in which the general principle may be said to be (*a*) the use of substances which, while increasing the staining power, tend to fix the stain in the bacteria, and (*b*) the subsequent treatment by substances which decolorise the overstained tissues to a greater or less extent, while they leave the bacteria coloured. The staining capacity of a solution may be increased:

(*a*) By the addition of substances such as carbolic acid, aniline oil, or metallic salts.

(*b*) By the addition of alkalis, such as caustic potash or ammonium carbonate, in weak solution.

(*c*) By the employment of heat.

(*d*) By long duration of the staining process.

As decolorising agents we use chiefly mineral acids (hydrochloric, nitric, sulphuric), vegetable acids (especially acetic acid), alcohol (either methylated spirit or absolute alcohol), or a combination of spirit and acid, *e.g.* methylated spirit with a drop or two of hydrochloric acid added, also various oils, *e.g.* aniline, clove, etc. In most cases about 30 drops of acetic acid in a bowl of water will be sufficient to remove the excess of stain from over-stained films and sections. More of the acid may, of course, be added if necessary. Hot water also decolorises to a certain extent; over-stained films can often be readily decolorised by placing a drop of water on the film and heating gently over a flame. When preparations have been sufficiently decolorised by an acid, they should be well washed in tap water, or in distilled water with a little lithium carbonate added.

Different organisms take up and retain the stains with various degrees of intensity, and thus duration of staining and decolorising must be modified accordingly.

When methylene-blue, crystal-violet, methyl-violet, or gentian-violet is used, the stain can, after the proper degree of decolorisation has been reached, be fixed in the tissues by treating for a minute with ammonium molybdate (2½ per cent. in water).

The Formulæ of some of the more commonly used Stain Combinations :

1. *Loffler's Methylene-blue.*

Saturated solution of methylene-blue in alcohol . . 30 c.c.
Solution of potassium hydrate in distilled water (1 : 10,000) 100 ,,

(The latter solution may be conveniently made by adding 1 c.c. of a 1 per cent. solution to 99 c.c. of water.)

Sections may be stained in this mixture for from a quarter of an hour to several hours. They do not readily over-stain. The tissue containing the bacteria is then decolorised if necessary with ½–1 per cent. acetic acid, till it is a pale blue-green. The section is washed in water, rapidly dehydrated with alcohol or aniline oil, cleared in xylol, and mounted.

The tissue may be counter-stained with eosin. If this is desired, after decolorisation wash with water, place for a few seconds in 1 per cent. solution of eosin in absolute alcohol, rapidly complete dehydration with pure absolute alcohol, and proceed as before.

Films may be stained with Löffler's blue by five minutes' exposure

or longer in the cold. They usually do not require decolorisation, as the tissue elements are not over-stained.

2. *Polychrome Methylene-blue.*—This is a methylene-blue solution which has been "ripened" by oxidation so that new violet compounds are formed which stain certain structures, *e.g.* the granules in the B. diphtheriæ. Such a solution may be conveniently made from Löffler's methylene-blue by shaking a half-filled bottle from time to time over a long period; several months are necessary to complete the process.

Unna's polychrome methylene-blue solution has the following composition:

Methylene-blue	1 gram.
Carbonate of potassium	1 ,,
Distilled water	100 c.c.

It is "ripened" by the method just described and for use is diluted with 5 to 10 volumes of water

3. *Kuhne's Methylene-blue.*

Methylene-blue	1·5 gram.
Absolute alcohol	10 c.c
Carbolic acid solution (1 : 20)	100 ,,

Stain and decolorise as with Loffler's blue, or decolorise with very weak hydrochloric acid (a few drops in a bowl of water).

4. *Carbol-Thionin-blue.*—Make up a stock solution consisting of 1 gram of thionin-blue dissolved in 100 c.c. carbolic acid solution (1 : 40). For use, dilute one volume with three of water, and filter. Stain sections for five minutes or upwards. Wash very thoroughly with water, otherwise a deposit of crystals may occur in the subsequent stages. Decolorise if necessary with very weak acetic acid. A few drops of the acid added to a bowl of water are quite sufficient. Wash again thoroughly with water. Dehydrate with absolute alcohol. Thionin-blue stains more deeply than methylene-blue, and gives equally good differentiation. It is very suitable for staining typhoid and glanders bacilli in sections. Cover-glass preparations stained by this method do not usually require decolorisation. As a contrast stain, 1 per cent. watery solution of eosin may be used before staining with the thionin.

5. *Gentian-violet in Aniline Oil Water.*—Two solutions have here to be made up. (*a*) Aniline oil water. Add about 5 c.c. aniline oil to 100 c.c. distilled water in a flask, and shake violently till as much as possible of the oil has dissolved. Filter and keep in a covered bottle to prevent access of light. (*b*) Make a saturated solution of gentian-violet in alcohol. When the stain is to be used, 1 part of (*b*) is added to 10 parts of (*a*), and the mixture filtered. The mixture should be made not more than twenty-four hours before use. Stain sections for a few minutes; then decolorise with methylated spirit. Sometimes it is advantageous to add to the methylated spirit a little hydrochloric acid (2–3 drops to 100 c.c.). This staining solution is not so much used by itself as in Gram's method, which is presently to be described.

6. *Ziehl-Neelsen Carbol-Fuchsin.*—This has the composition:

Basic fuchsin	1 gram.
Alcohol	10 c.c.
Carbolic acid solution (1 : 20)	100 ,,

The fuchsin is dissolved in alcohol and the solution is then added to the solution of carbolic acid. This is a very powerful stain, and, when used in the undiluted condition, a few seconds' staining is usually sufficient. It is better, however, to dilute with from five to ten times its volume of water and stain for a few minutes. In this form it has a very wide application. Methylated spirit with or without a few drops of acetic acid is the most convenient decolorising agent.

Gram's Method and its Modifications.—In the methods already described, the tissues, and more especially the nuclei, retain some stain when decolorisation has reached the point to which it can safely go without the bacteria themselves being affected. In the method of Gram, now to be detailed, this does not occur, for *the stain can here be removed completely from the ordinary tissues, and left only in the bacteria.* The stain used is a violet, and, according to Unna, Gram's method can be carried out only with the pararosanilin group of dyes (*e.g.* methyl-violet, crystal-violet, or gentian-violet which is a mixture of the first two). The essential step in the method is the treatment of the preparation with a solution of iodine after staining. The iodine forms with the stain in the bacteria a compound which resists decolorisation with such agents as alcohol, aniline-xylol, acetone, etc., whilst the tissues are decolorised and can then be coloured by a contrast stain. All kinds of bacteria, however, do not retain the stain in this method, and therefore in the systematic description of any species it is customary to state whether or not it retains the violet stain in Gram's method—in other words, whether it is *Gram-positive* or *Gram-negative.* It must also be mentioned that some tissue elements may retain the stain as firmly as any bacteria, *e.g.* keratinised epithelium, calcified particles, the granules of mast cells, and sometimes altered red blood corpuscles, etc.

Various modifications of Gram's original method have been introduced, these depending chiefly on the formula of the stain and the decolorising agent used. The following have been selected as suitable :

Weigert's Modification of Gram's Method

The following are the solutions necessary :

(1) Carbol-gentian-violet —

Saturated alcoholic solution of gentian-violet	1 part
Carbolic acid solution (1 : 20) . . .	10 parts.

(2) Gram's iodine—

Iodine	1 gram
Potassium iodide	2 grams.
Distilled water	300 c.c.

(3) Aniline-oil-xylol—

Aniline oil	2 parts.
Xylol	1 part.

(4) Dilute carbol-fuchsin—

Carbol-fuchsin (p. 105)	1 part.
Distilled water	9 parts

A. FILMS after being fixed are treated as follows :

(1) Stain with carbol-gentian-violet for two to three minutes.

(2) Pour off stain and, without washing, add Gram's iodine ; allow to act for about a minute.

(3) Pour off iodine and dry with blotting paper, afterwards heating the preparation slightly (it is important that the preparation should be thoroughly dried).

(4) Decolorise with aniline-xylol till the preparation is of a pale violet colour. In the case of films of pus, etc., or sections, decolorising should be controlled under the low power of the microscope till the nuclei have a faint violet colour.

(5) Wash off aniline-xylol thoroughly by means of xylol and then allow to dry.

(6) Counter-stain with dilute carbol-fuchsin for about ten to twenty seconds. Wash in water and dry.

Control Spot.—In order to avoid errors from over-decolorising when examining pus for Gram-negative organisms, it is advisable to make a small film of a known Gram-positive organism (from a twenty-four hours' pure culture, *e.g.* of staphylococcus) at one side of the pus smear ; this " control spot " is then treated in the same way as the rest of the film. For the *diagnosis of Gram-negative organisms*, e g. *gonococci or meningococci in pus*, it is essential that the " control spot " should retain the Gram's stain vigorously, while the nuclei of the pus cells are at the same time stained pink.

B. SECTIONS should be first stained by carmalum for ten minutes. The steps of procedure are then the same as the above, but in stage (5), after being treated with xylol, the preparation is mounted in balsam.

Jensen's Modification of Gram's Method

This is rapidly carried out and gives good results.

Solutions required :

(1) Methyl-violet (6 B)	0·5 gram
Water	100 c.c.
(2) Iodine solution—	
Iodine	1 gram.
Potassium iodide	2 grams.
Water	100 c.c,
(3) Contrast stain—	
Neutral-red	1 gram.
Acetic acid (1 per cent.)	2 c.c.
Water	1000 ,,

Films or sections are treated as follows :

(1) Stain with violet solution for a quarter to a half minute.

(2) Wash off stain with the iodine solution and allow this to act for a half to one minute.

(3) Wash off iodine solution with absolute alcohol and treat with fresh alcohol until the preparation is sufficiently decolorised.

(4) Wash off the alcohol with the neutral-red solution and allow the counter-stain to act for about half a minute.

(5) Wash with water. *Films* are blotted with filter paper and dried. *Sections* are dehydrated with absolute alcohol, cleared in xylol, and mounted.

Burke's Modification of Gram's Method

A. FILMS are allowed to dry in air, fixed with the least amount of heat necessary, and treated as follows :

(1) Flood with 1 per cent. watery crystal-violet solution, then mix with the dye on the slide 3 to 8 drops of a fresh 5 per cent solution of sodium bicarbonate, and allow to stand for two to three minutes.

(2) Flush off the excess of stain wth iodine solution (1 gram iodine, 2 grams potassium iodide, 100 c.c. distilled water), then cover with fresh iodine solution, and let stand one minute or longer.

(3) Wash in water quickly.

(4) Blot off all free water ; but do not dry.

(5) Decolorise with acetone (or acetone 3 parts and ether 1 part) until the decoloriser flows from the slide practically uncoloured (this usually takes less than ten seconds).

(6) Blot dry.

(7) Counter-stain for five to ten seconds or longer with a 2 per cent. watery solution of safranin 0 (dilute carbol-fuchsin—1 : 10 may be used instead).

(8) Wash in water, blot, and dry.

B. SECTIONS (according to Dunlop) are treated as above at stages (1) to (5) ; then one proceeds as follows :

(*a*) Wash in water.

(*b*) Counter-stain with Ziehl-Neelsen carbol-fuchsin diluted 1 : 250 with water, for two minutes.

(*c*) Wash in water.

(*d*) Dehydrate quickly with methylated spirit followed by absolute alcohol.

(*e*) Clear in xylol and mount in balsam.

There is great variability in the avidity with which organisms stained by Gram retain the dye when washed with alcohol, or other decolorising agent, and sometimes difficulty is experienced in saying whether an organism does or does not stain by this method. Most bacteria are either frankly Gram-positive or frankly Gram-negative, but cases occur where an organism, usually Gram-positive or Gram-negative, tends when grown on certain media to show an opposite tendency, and sometimes an organism is met with in which the individuals in a film show slightly different reactions to the Gram stains. The commonest variation is for a Gram-positive organism to become in older cultures Gram-negative.

Stain for Tubercle and other Acid-fast Bacilli.—These bacilli cannot be well stained with a simple watery solution of a basic aniline dye. This fact can easily be tested by attempting to stain a film of a tubercle culture with such a solution; with the Gram method, however, a partial staining is effected. Such bacteria require a powerful stain containing a mordant, and must be exposed to the stain for a long time, or its action may be aided by a short application of heat. When once stained, however, they resist decolorising even with very powerful acids; they are therefore called "acid-fast." The smegma bacillus also resists decolorising with strong acids (p. 331), and a considerable number of other acid-fast bacilli are now known (p. 329). Any combination of crystal-violet or fuchsin with aniline oil or carbolic acid or other mordant will stain the bacilli named, but the following method is most commonly used:

1. Filter a few drops of Ziehl-Neelsen carbol-fuchsin (p. 105) on to the film or section, heat till steam rises, and repeat this several times, allowing the stain to act for five minutes; or place the preparation in the cold, stain for twelve to twenty-four hours. (Films and paraffin sections are usually stained with hot stain, loose sections with cold; in hot stain the latter shrink.)
2. Decolorise with 20 per cent. solution of strong sulphuric acid, nitric acid, or hydrochloric acid, in water. In this the tissues become yellow.
3. Wash well with water. The tissues will regain a faint pink tint. If the colour is distinctly red, the decolorisation is insufficent, and the specimen must be returned to the acid. As a matter of practice, it is best to remove the preparation from the acid every few seconds and wash in water, replacing the specimen in the acid and re-washing till the proper pale pink tint is obtained. Then wash in alcohol for half a minute, and replace in water.
4. Contrast stain with a saturated watery solution of methylene-blue for half a minute.
5. Wash well with water. In the case of *films*, blot and dry. In the case of *sections*, dehydrate, clear, and mount.

Leprosy bacilli are stained in the same way, but are rather more easily decolorised than tubercle bacilli, and it is better to use only 5 per cent. sulphuric acid in decolorising.

In the case of specimens stained by the Ziehl-Neelsen method the tubercle or leprosy bacilli ought to be bright red, and the tissue blue. Other bacteria which may be present are also coloured with the blue stain.

Stains for Diphtheria Bacillus.—The following methods for bringing out the structural characters of this organism may be recommended. In the case of cultures, films should be made from eighteen to twenty-four hours' growth on serum medium.

Neisser's Stain (second or modified method)

1. Stain films for a few seconds in a mixture of solutions A and B, two parts of the former to one of the latter.

A.	Methylene-blue	1 gram.
	Absolute alcohol	50 c.c.
	Glacial acetic acid	50 ,,
	Distilled water	1000 ,,
B.	Crystal-violet (Höchst)	1 gram.
	Absolute alcohol	10 c.c.
	Distilled water	300 ,,

2. Wash in water, and
3. Stain in chrysoidin solution (1 : 300) for a few seconds (the chrysoidin should be dissolved in warm water and the solution then filtered).
4. Wash in water, blot, and dry.

The substance of the bacilli is brownish yellow, the granules are almost black.

Instead of chrysoidin the following solution of erythrosin may be used with advantage: Saturated alcoholic solution of erythrosin, 20 parts; saturated watery solution of picric acid, 90 parts; add to the mixture precipitated calcium carbonate to excess; allow to stand for a time, shaking at intervals; filter.

Pugh's Stain

The staining solution consists of

Toluidin blue, 10 per cent. solution in absolute alcohol	2 c.c.
5 per cent. solution of glacial acetic acid in distilled water	100 ,,

Stain for two to three minutes. Heat till steam rises. Wash with water and dry. The substance of the diphtheria bacilli is light blue and the granules are of a reddish purple tint.

The Staining of Spores.—If bacilli containing spores are stained with a watery solution of a basic aniline dye the spores remain unstained. The spores either take up the stain less readily than the protoplasm of the bacilli, or they have a resisting envelope which prevents the stain from penetrating to the protoplasm. Like tubercle bacilli, when once stained they retain the colour with considerable tenacity. In fixing the films little heat should be used, as overheating interferes with the subsequent staining. The following is the simplest method for staining spores:

1. Stain films with carbol-fuchsin (p. 105), heating as for tubercle bacilli.
2. Decolorise with 1 per cent. sulphuric acid in water or with methylated spirit. This removes the stain from bacilli.

3. Wash in water.
4. Stain with saturated watery methylene-blue for half a minute.
5. Wash in water, and dry.

The result is that the spores are stained red, the protoplasm of the bacilli blue. (In some cases decolorising as above is unnecessary, the treatment with methylene-blue effecting sufficient decolorisation.)

The spores of some organisms lose the stain more readily than those of others, and for staining some, methylated spirit is a sufficiently strong decolorising agent to use. If sulphuric acid stronger than 1 per cent. is used, the spores of many bacilli are readily decolorised

Moller's Method.—The following method, recommended by Möller, is much more satisfactory than the previous. Before being stained, the films are placed in chloroform for two minutes, and then in a 5 per cent solution of chromic acid for a half to two minutes, the preparation being well washed after each reagent. Thereafter they are stained and decolorised as above.

The Staining of Capsules.—The following methods may be recommended in the case of capsulated bacteria :

1. *Hiss's Method*

In this method the staining solution consists of 1 part of a saturated alcoholic solution of fuchsin or gentian-violet and 19 parts of distilled water. A few drops of the stain are placed on a film, previously dried and fixed by heat, and the preparation is steamed for a few seconds over a flame. The staining solution is washed off with a 20 per cent solution of copper sulphate; the preparation (without being washed in water) is dried between filter papers The capsules of pneumococci in exudates or growing in a fluid serum medium can be readily demonstrated by this method ; in the case of solid cultures, films should be made without any diluent, or a drop of fluid serum should be used The method is easily applied, and gives excellent results.

2. *Richard Muir's Method* (modified)[1]

1. The film containing the bacteria must be very thin. It is dried and stained in filtered carbol-fuchsin for half a minute, the preparation being gently heated
2. Wash slightly with spirit and then well in water.
3. Place in following mordant for a few seconds :

Saturated solution of corrosive sublimate .	2 parts.
Tannic acid solution 20 per cent . .	2 ,,
Saturated solution of potash alum . .	5 ,,

4. Wash well in water.
5. Treat with methylated spirit for about a minute.

The preparation has a pale reddish appearance.

[1] For a method of staining capsules of bacteria in sections, reference may be made to Rd. Muir, *Journ. Path. and Bact.*, xx. (1916), 257.

6. Wash well in water.
7. Counter-stain with watery solution of ordinary methylene-blue for half a minute
8. Dehydrate in alcohol, clear in xylol, and mount in balsam.

The bacteria are a deep crimson, and the capsules of a blue tint. The capsules of bacteria in certain culture media may be demonstrated by this method.

3. *J. Kirkpatrick's Method*

Solutions required :

(1) Fixing solution—

Formalin	10 c.c.
Chloroform	30 ,,
Absolute alcohol	70 ,,

(2) Manson's borax methylene-blue—

Methylene-blue	1 gram
Borax	2 grams.
Water	100 c.c.

Dissolve borax in boiling water over flame, add methylene-blue, cool, and filter. For use take

Borax methylene-blue	1 part.
Distilled water	5 parts.

(1) Make film on slide, then allow to dry in air (the film must not be heated).

(2) Pour on fixing solution and allow to remain for one to three minutes.

(3) Treat with spirit. Wash in water and stain with dilute borax methylene-blue for one to three minutes.

(4) Wash in water, blot, and dry ; or dehydrate in acetone, clear in xylol, and mount in balsam.

4. Capsules can also be demonstrated by the indian-ink method (p. 115).

The Staining of Flagella.—The staining of the flagella of bacteria is the most difficult of all bacteriological procedures, and it requires considerable practice to ensure that good results shall be obtained. Many methods have been introduced, of which the two following are very satisfactory :

Preparation of Films.—In all the methods of staining flagella, young cultures on agar should be used, say a culture incubated for from ten to eighteen hours at 37° C. A very small portion of the growth is taken on the point of a platinum needle, and carefully mixed in a little water in a watch-glass ; the amount should be such as to produce scarcely any turbidity in the water. A film is then made by placing a drop on a clean cover-glass and carefully spreading it out with the needle. It is allowed to dry in the air, and is then passed twice or thrice through a flame, care being taken not to over-heat it. The cover-glasses used

should always be cleaned in the mixture of sulphuric acid and potassium bichromate described on page 99, and must be scrupulously clean.

1. *Pitfield's Method as modified by Richard Muir*

Prepare the following solutions :

A. *The Mordant.*

Tannic acid, 10 per cent. watery solution, filtered	10 c.c.
Corrosive sublimate, saturated watery solution	5 ,,
Alum, saturated watery solution	5 ,,
Carbol-fuchsin (*vide* p. 105)	5 ,,

Mix thoroughly. A precipitate forms, which must be allowed to deposit, either by centrifugalising or simply by allowing to stand. Remove the clear fluid with a pipette, and transfer to a clean bottle. The mordant keeps well for one or two weeks

B. *The Stain.*

Alum, saturated watery solution	10 c.c.
Gentian-violet, saturated alcoholic solution	2 ,,

The stain should not be more than two or three days old when used. It may be substituted in the mordant in place of the carbol-fuchsin.

The film having been prepared as above described, pour over it as much of the mordant as the cover-glass will hold Heat gently over a flame till steam begins to rise, allow to steam for about a minute, and then wash well in a stream of running water for about two minutes. Then dry carefully over the flame, and when thoroughly dry pour on some of the stain. Heat as before, allowing to steam for about a minute, wash well in water, dry, and mount in a drop of xylol balsam.

2. *J. Kirkpatrick's Method*

Solutions required :

Fixing solution (Zenker's without acetic acid)

Potassium bichromate	2·5 grams
Sodium sulphate	1 gram.
Perchloride of mercury	5 grams
Water	100 c.c.

Mordant

Tannic acid, 20 per cent. watery solution	3 parts.
Ferric chloride, 5 per cent. watery solution	1 part

Silver Solution

Distilled water	5 c.c.
Silver sulphate (cold saturated watery solution)	15 ,,

Place 5 c.c. of distilled water in a large test-tube, add silver sulphate solution, and mix thoroughly. From a pipette add 33 per

cent. ethylamine drop by drop till the turbidity produced at first is just dissolved. The resulting solution should be slightly opalescent. Excess of ethylamine makes the solution clear; it is then useless. If there has been excess of ethylamine additional silver sulphate may be added.

Films are teated as follows :

(1) Make film on slide from a young culture ; allow to dry in air.
(2) Fix in fixing solution for one to three minutes.
(3) Wash in water.
(4) Treat with mordant for five minutes or longer.
(5) Wash in water
(6) Cover slide with silver solution and heat gently until film becomes a faint brown colour - half to one minute.
(7) Run stream of water directly on to slide for half a minute. (The silver solution must not be poured off and the film then washed, as deposits are then liable to occur)
(8) Stain with dilute carbol-fuchsin (1 : 10) for one minute.
(9) Wash, dry, and mount in neutral Canada balsam.

Staining of Spirochætes in Sections.—The following impregnation methods have been applied for this purpose by Levaditi, and give excellent results :

1. *Levaditi's Original Method*

(1) The tissues, which ought to be in thin slices, about 1 mm. in thickness, are best fixed in 10 per cent. formalin solution for twenty-four hours.

(2) They are washed for an hour in water, and then brought into 96 per cent. alcohol for twenty-four hours.

(3) They are then placed in 1 5 per cent. solution of nitrate of silver in a dark bottle, and are kept in an incubator at 37° C. for three days.

(4) They are washed in water for about twenty minutes, and are thereafter placed in the following mixture, namely :

Pyrogallic acid	4 grams.
Formalin	5 c c.
Distilled water up to . . .	100 ,, .

They are kept in this mixture in a dark bottle for forty-eight hours at room temperature.

(5) They are then washed in water for a few minutes, taken through increasing strengths of alcohol, and embedded in paraffin in the usual way. The sections, which ought to be as thin as possible, are fixed on slides as usual ; the paraffin is removed by xylol and the preparation is mounted in balsam. In satisfactory preparations the spirochætes appear of an almost black colour against the pale yellow background of the tissues. The latter can be contrast-stained by weak carbol-fuchsin or by toluidin blue.

2. *Levaditi's Newer Pyridin Method*

(1) The tissues are fixed in formalin as in the previous method, are hardened in alcohol for twelve to sixteen hours, and then washed in water.

(2) They are then impregnated with a 1 per cent. solution of silver nitrate, to which 10 per cent. of pyridin puriss is added at the time of use. The tissues are placed in the solution in a well-stoppered bottle, and are kept for two to three hours at room temperature and four to six hours at about 50° C. They are thereafter washed quickly in 10 per cent. pyridin solution.

(3) Reduction is then carried out in the following mixture, namely, a 4 per cent. solution of pyrogallic acid to which are added, at the time of use, 10 per cent. pure acetone and 15 per cent. pyridin.

(4) The tissues are then put through alcohol and xylol, and embedded in paraffin. The sections can be stained with toluidin blue or Unna's polychrome blue.

This method, though more rapid than the previous, is suitable only for fresh tissues. For ordinary post-mortem material Levaditi's original method is to be recommended.

Examination of Spirochætes in Films.—The following methods may be recommended :

1. *Fontana's Method*

This is a silver impregnation method, and three solutions are necessary—a fixing fluid, a mordant, and a silver solution. They are as follows :

(*a*) Acetic acid 1 c.c., formalin 20 c.c., and water 100 c.c.

(*b*) 5 per cent. tannic acid in a 1 per cent. watery solution of carbolic acid.

(*c*) ¼ per cent solution of silver nitrate in distilled water. For use a small quantity of this is put in a test-tube, and a minute amount of ammonia solution is added till there is distinct turbidity. (If too much ammonia is added the fluid becomes clear again.)

Dried films, which should not be fixed by heat, are fixed in solution (*a*) for about a minute, the fluid being dropped on the film and renewed once or twice. The preparation is then washed thoroughly in running water, solution (*b*) is dropped on the film, heated till steam rises, and allowed to remain for about half a minute. It is again washed in water, solution (*c*) is dropped on, heated till steam rises, and allowed to remain for another half minute. The preparation is finally washed in water and dried.

The spirochætes are of a dark brown or black colour, and are easily found. This is an excellent method, and is easily carried out.

2. *Indian Ink and Collargol Method*

In the first, an emulsion of indian ink of fine quality is sterilised by steaming and allowed to settle for a few days ; a drop of the deposit diluted with an equal quantity of distilled water is well rubbed up and spread on a slide with a drop of the material to be examined (exudate from chancre or condyloma, scraping from congenitally affected organ, etc.). The film is dried and examined with an immersion lens without the interposition of a cover. Spirochætes, if present, stand out unstained, surrounded by the dark indian ink, and often positive results are rapidly obtained by means of it. The organisms are not so readily recognised by this method

as by dark-ground illumination, and negative observations are thus less valuable.

Harrison, in place of indian ink, uses a suspension of collargol in distilled water (1 : 19), which should be shaken before use. It is employed in a similar way.

3. *Becker's Method*

(*a*) Treat thin, dried, but unfixed, films for one minute with a solution consisting of glacial acetic acid 1 c.c., formalin 2 c.c., distilled water 100 c.c. The fluid is renewed twice in the course of the treatment. (*b*) Rinse in distilled water. (*c*) Mordant with a solution containing : carbolic acid 1 gram, tannic acid 10 grams, distilled water 100 c c., warming gently till steam rises for half a minute. (*d*) Rinse in water. (*e*) Stain with warm Ziehl-Neelsen carbol-fuchsin solution for half to three-quarters of a minute. (*f*) Rinse well in water, blot, and dry.

4. *Giemsa's Stain* (p. 118)

The following is a rapid method suitable for spirochætes. The film is fixed in absolute alcohol for fifteen minutes or by drawing three times through the flame. Immediately before use 10 drops of Giemsa's solution are added to 10 c.c water and the mixture is shaken gently. The film is covered with the diluted stain and warmed till steam rises ; after fifteen seconds this is poured off and fresh stain added, the film is again heated ; this procedure is repeated four or five times. Finally the film is washed in water and dried.

The Romanowsky Stains.—Within recent years the numerous modifications of the Romanowsky stain have been extensively used. The dye concerned is the compound which is formed when watery solutions of medicinal methylene-blue and water-soluble eosin are brought together. This compound is insoluble in water but soluble in alcohol—the alcohol employed being methyl alcohol. The stain was originally used by Romanowsky for the malarial parasite, and its special quality is that it imparts to certain elements, such as the chromatin of this organism, a reddish-purple hue. This was at first thought to be simply due to the combination of the methylene-blue and the eosin, but it is now recognised that certain changes, such as occur in methylene-blue solutions with age, are necessary. In the modern formulæ these changes are brought about by treatment with alkalis, especially alkaline carbonates, as was first practised by Unna in the preparation of his polychrome methylene-blue. The stains in use thus contain a mixture of methylene-blue and its derivatives in combination with eosin ; the differences in these bodies and the different proportions in which they occur in individual stains account for the different effects produced on the various constituents of a cell. The

underlying chemical reactions are complicated and as yet not fully understood. Thus it is not certainly known to what particular new body the reddish hue produced in chromatin is due, but the active constituent may be methylene-violet or methyl-azure, or thionin, all of which result from the action of alkali on methylene-blue. The stains are much used in staining blood films (in which the characters of both nucleus and cytoplasm in leucocytes are beautifully brought out), in staining bacteria in tissues or exudates, the malaria parasite, trypanosomes, the pathogenic spirochætes (such as the Treponema pallidum), and protozoa generally.

The following are the chief formulæ in use :

1. *Jenner's Stain.*—This is an excellent blood stain, but is not so good for the study of parasites as the others to be mentioned. The stain is supplied as a powder, and of this 0·5 gram is dissolved in 100 c.c. Merck's methyl alcohol. For use a few drops are placed on the dried unfixed film for one to three minutes, the dye is poured off, and the preparation washed with distilled water till it presents a pink colour ; it is then dried between filter paper and mounted in xylol balsam.

2. *Leishman's Stain* —This is supplied as a powder or as a solution already made up. Of the powder, 0·15 per cent. is dissolved in Merck's methyl alcohol (" for analysis, acetone free ") as follows : The powder is placed in a clean mortar, a little of the alcohol is added and well rubbed up with a pestle ; the undissolved powder is allowed to settle, and the fluid decanted into a dry bottle ; the process is repeated with fresh fractions of the solvent till practically all the stain is dissolved, and the bottle is well stoppered. The stain will keep for a long period.

Method.—Blood films are allowed to dry before being stained.

(1) Support the slide or cover-glass in a horizontal position and raised off the bench.

(2) Drop on the stain from a pipette until the whole surface is uniformly covered (5–10 drops for a cover-glass ; 20–30 drops for a slide). Allow to act for one and a half minutes.

(3) With the same pipette, add 2 drops of distilled water (or soft tap water) for each drop of stain used under (2). The fluids will mix thoroughly without any rocking of the slide or cover-glass, and at least one quarter of an hour, and preferably a half to one hour or longer, is allowed for staining to be effected.

(4) Do not pour off the stain, but allow a gentle stream of water (*soft* tap water, or distilled water) to wash it away. The film should appear bright pink in colour, and the washing may be continued for a half to two minutes.

(5) Drain off excess of water and allow the stained film to dry in air (do not heat).

(6) Mount in neutral Canada balsam.

The results are most satisfactory with freshly prepared films or with such as are only a few days old. If older than this, there is an increasing tendency for the red cells to stain of a dusky bluish colour or else for them to remain practically unstained while the

surrounding dried plasma exhibits markedly basophile characters. It is to be noted, however, that malarial parasites, spirochætes, etc., remain readily recognisable in spite of such altered staining affinities on the part of the red blood corpuscles.

The colour of red cells may be restored by washing the film with acetic acid, 1 : 1500, after staining.

For staining sections a little modification is necessary. A paraffin section is taken into distilled water as usual, the excess of water is drained off, and a mixture of one part of stain and two parts of distilled water is placed on it. The stain is allowed to act for five to ten minutes till the tissue appears a deep Oxford blue ; it is then decolorised with 1 : 1500 acetic acid—the effect being watched under a low-power lens. The blue begins to come out, and the process is allowed to go on till only the nuclei remain blue. The section is then washed with distilled water, rapidly dehydrated with alcohol, cleared, and mounted. If, as sometimes happens, the eosin tint be too well marked, it can be lightened by the action of 1 : 7000 solution of caustic soda, this being washed off whenever the desired colour has been attained.

In certain cases, *e g.* for the staining of old films or of trypanosomes or Leishmaniæ in sections, Leishman recommends an initial treatment of the preparation with serum. This modification is described below (p. 676).

3. *J. H. Wright's Stain.*—This stain, which is much used in America, is applied in almost the same way as Leishman's. A few drops of the solution in methyl alcohol are placed on the preparation for a minute for fixation ; water is then dropped on till a green iridescent scum appears on the top of the fluid, and staining goes on for about two minutes ; the stain is then washed off with distilled water, and a little is allowed to remain on the film till differentiation is complete ; the preparation is carefully dried with filter paper.

4. *Giemsa's Stain.*—Giemsa believes that the reddish-blue hue characteristic of the Romanowsky stain is due to the formation of methyl-azure, and he has prepared this by a method of his own under the name " Azur I." From this, by the addition of an equal part of medicinal methylene-blue, he prepares what he calls " Azur II.," and from this again by the addition of eosin he prepares "Azur II.-eosin." The latest formula for the finished stain is as follows : Azur II.-eosin, 3 grams ; Azur II., 0·8 gram ; glycerin (Merck, chemically pure), 250 grams ; methyl alcohol (Kahlbaum, I.), 250 grams. This stain has been extensively used for demonstrating spirochætes, but it can be used for any other purpose to which the Romanowsky stains are applicable. For spirochætes the following are Giemsa's directions :

(1) Fix films in absolute alcohol for fifteen to twenty minutes, dry with filter paper. (2) Dilute stain with distilled water—one drop of stain to 1 c.c. water (the mixture being well shaken). (Sometimes the water is made alkaline by the addition of one drop of 1 per cent. potassium carbonate to 10 c.c. water.) (3) Stain for fifteen minutes (a longer period is often desirable, even twenty-four hours). (4) Wash in brisk stream of distilled water. (5) Drain with filter paper, dry, and mount in Canada balsam.

Sabouraud's Method for Staining Trichophyta. — Remove the fat from the hair or epithelial squames with chloroform. Place in a test-tube with 10 per cent. formol, and warm for two or three

minutes till ebullition commences. Wash well in distilled water, and stain for one minute in Sahli's blue, which is made up as follows:

Distilled water	40 parts
Saturated watery methylene-blue .	24 ,,
5 per cent. solution of borax in water	16 ,,

Mix the constituents. Allow to stand for a day, and filter

After staining, wash in water, dehydrate with absolute alcohol, clear in xylol, and mount in balsam.

The Fixation and Hardening of Tissues.—The following are amongst the best methods for bacteriological purposes:—

(*a*) *Absolute alcohol* may be used for the double purpose of fixing and hardening. If the piece of tissue is not more than $\frac{1}{8}$ inch in thickness, it is sufficient to keep it in this reagent for twenty-four hours. If the pieces are thicker, a longer exposure is necessary, and in such cases it is better to change the alcohol at the end of the first twenty-four hours. If the tissues are not to be cut at once, they may be preserved in 50 per cent. spirit.

(*b*) *Formalin Solution.*—This may be used as a 10 per cent. solution of commercial formaldehyde in water. Thin pieces of tissue are fixed in this for twenty-four hours; they are then washed in water for a few hours and placed in 50 per cent spirit for twenty-four hours, and then in pure spirit

(*c*) *Corrosive sublimate* is an excellent fixing agent It is best used as a saturated solution in 0·75 per cent. sodium chloride solution. Dissolve the sublimate in the salt solution by heat; the separation of crystals on cooling shows that the solution is saturated. For small pieces of tissue $\frac{1}{8}$ inch in thickness, twelve hours' immersion is sufficient. If the pieces are larger, twenty-four hours is necessary. They should then be tied up in a piece of gauze, and placed in a stream of running water for from twelve to twenty-four hours, according to the size of the pieces, to wash out the excess of sublimate. They are then placed for twenty-four hours in each of the following strengths of methylated spirit (free from naphtha [1]): 30 per cent., 60 per cent., and 90 per cent. Finally they are placed in absolute alcohol for twenty-four hours, and are then ready to be prepared for cutting.

If the tissue is very small, as in the case of minute pieces removed for diagnosis, the stages may be all compressed into twenty-four hours. In fact, after fixation in corrosive the tissue may be transferred directly to absolute alcohol, the perchloride of mercury being removed by Gram's iodine solution after the sections are cut (p. 100).

(*d*) *Zenker's Fluid*—Potassium bichromate, 2·5 grams; sodium sulphate, 1 gram; corrosive sublimate, 5 grams; water, 100 c.c. Before use add 5 c.c. of glacial acetic acid to 100 c.c. of fixing fluid. Pieces of tissue should be small and fixed for not more than eighteen

[1] In Britain ordinary commercial methylated spirit has mineral naphtha added to it to discourage its being used as a beverage. The naphtha being insoluble in water a milky fluid results from the dilution of the spirit. By law, chemists can only sell 8 ounces of pure spirit at a time. Most pathological laboratories are, however, permitted by the Excise to buy "industrial spirit," which contains only one-nineteenth of wood naphtha.

to twenty-four hours. They are then treated as after fixation with corrosive sublimate.

The Cutting of Sections.—A. *By Means of the Freezing Microtome.*—Pieces of tissue hardened by any of the above methods must have all the alcohol removed from them by washing in running water for twelve to twenty-four hours. They are then placed for twelve to twenty-four hours (according to their size) in a thick syrupy solution containing 2 parts of gum arabic and 1 part of sugar. They are then cut on a freezing microtome and placed for a few hours in a bowl of water so that the gum and syrup may dissolve out. They are then stained, or they may be stored in methylated spirit.

B. *Embedding and Cutting in Solid Paraffin.*—This method gives by far the finest results, and should always be adopted when practicable. The principle is the impregnation of the tissue with paraffin in the melted state. This paraffin when it solidifies gives support to all the tissue elements. The method involves that, after hardening, the tissue shall be thoroughly dehyrated, and then thoroughly permeated by some solvent of paraffin which will expel the dehydrating fluid and prepare for the entrance of the paraffin. The solvents most in use are chloroform, cedar oil, xylol, and turpentine; of these, chloroform is the most suitable. The more gradually the tissues are changed from reagent to reagent in the processes to be gone through, the more successful is the result. A necessity of the process is an oven, in which the paraffin can be kept at a constant temperature just above its melting-point, a regulator being of course necessary. The tissues occurring in pathological work have at endency to become brittle if over-heated, and therefore the best results are obtained by using paraffin melting at a somewhat low temperature. We have used for some years a mixture of 1 part of paraffin melting at 48°, and 2 parts of paraffin melting at 54° C. This mixture has a melting-point between 52° and 53° C., and it serves all ordinary purposes well. An excellent quality of paraffin is that known as the "Cambridge paraffin," but many scientific-instrument makers supply paraffins which, for ordinary purposes, are quite as good, and much cheaper. The successive steps in the process of paraffin embedding are as follows:

1. Pieces of tissue, after having been hardened and brought through spirit are placed in fresh absolute alcohol for twenty-four hours in order to their complete dehydration.

2. Transfer now to a mixture of equal parts of absolute alcohol and chloroform for twenty-four hours.

3. Transfer to pure chloroform for twenty-four hours or longer. At the end of this time the tissues should sink or float heavily.

4. Transfer now to a mixture of equal parts of chloroform and paraffin and place in the paraffin oven for from twelve to twenty-four hours.

5. Place in pure melted paraffin in the oven for twenty-four hours. For holding the paraffin containing the tissues, small tin dishes such as are used by pastry-cooks will be found very suitable. There must be a considerable excess of paraffin over the bulk of tissue present, otherwise sufficient chloroform will be present to vitiate the final result and not give the perfectly hard block obtained with pure paraffin. With experience, the persistence of the slightest trace of chloroform can be recognised by smell.

In the case of very small pieces of tissue the time given for each

stage may be much shortened, and where haste is desirable Nos. 2 and 4 may be omitted. Otherwise it is better to carry out the process as described.

6. Cast the tissues in blocks of paraffin as follows: Pairs of L-shaped pieces of metal made for the purpose by instrument makers must be at hand. By laying two of these together on a glass plate, a rectangular trough is formed. This is filled with melted paraffin taken from a stock in a separate dish. In it is immersed the piece of tissue, which is lifted out of its pure paraffin bath with heated forceps. The direction in which it is to be cut must be noted before the paraffin becomes opaque. When the paraffin has begun to set, the glass plate and trough have cold water run over them. When the block is cold, the metal L's are broken off, and, its edges having been pared, it is stored in a pill-box.[1]

The Cutting of Paraffin Sections.—Sections must be cut as thin as possible, the Cambridge rocking microtome being, on the whole, most suitable. They should not exceed 8 μ in thickness and ought, if possible, to be about 4 μ. When cut, sections are floated on the surface of a beaker of water kept at a temperature about 10° C. below the melting-point of the paraffin. On the surface of the warm water they become perfectly flat. They are then fixed on slides.

Fixation on Ordinary Slides.—(*a*) *Gulland's Method.*—A supply of slides well cleaned being at hand, one of them is thrust obliquely into the water below the section, a corner of the section is fixed on it with a needle, and the slide withdrawn. The surplus of water being wiped off with a cloth, the slide is placed on a support, with the section downwards, and allowed to remain on the top of the paraffin oven or in a bacteriological incubator for from twelve to twenty-four hours. It will then be sufficiently fixed on the slide to withstand all the manipulation necessary during staining and mounting.

Sections of tissues fixed in Zenker's fluid or other bichromate solution cannot be fixed to slides by this method; albuminised slides, as in Mann's method, should be used.

(*b*) *Fixation by Mann's Method.*—This has the advantage of being more rapid than the previous one. A solution of albumin is prepared by mixing the white of a fresh egg with 10 parts of distilled water and filtering. Slides are made perfectly clean with alcohol. One is dipped into the solution and its edge is then drawn over one surface of another slide so as to leave on it a thin film of albumin. This is repeated with the others. As each is thus coated it is leant, with the film downwards, on a ledge till dry, and then the slides are stored in a wide stoppered jar till needed. The floating out is performed as before. The albuminised side of the slide is easily recognised by the fact that if it is breathed on, moisture does not condense on it. The great advantage of this method is that the section can be fixed after twenty to thirty minutes' drying at 37° C.

[1] While the method given is sufficient for ordinary purposes, a more elaborate technique is necessary if it is desired that no changes shall take place in the tissue. Thus after fixation the tissue must be taken up to absolute alcohol through successive dilutions of spirit, not differing from each other by more than 10 per cent. Again, when alcohol has been replaced by chloroform the latter must be saturated with chips of paraffin, first at room temperature, then at 37° C., and must be kept at 55° C. as short a time as possible.

CHAPTER IV

METHODS OF EXAMINING THE PROPERTIES OF SERUM—PREPARATION OF VACCINES—INOCULATION OF ANIMALS—METHODS OF OBTAINING PATHOLOGICAL MATERIAL FOR EXAMINATION

The Testing of Agglutinative and Sedimenting Properties of Serum

In studying the properties of serum it is necessary to have the means of measuring and diluting small quantities of fluid. The simplest method is by means of 1 c.c. and 0·1 c.c. pipettes.[1] Each pipette should be graduated in tenths, and should deliver to the end. If the original amount of fluid to be used is small, say less than 0·02 c.c., it should be diluted till it has fully this volume. This may be done by drawing up the fluid in a capillary tube (a piece of quill glass tubing drawn out in the flame being convenient for the purpose) and marking the upper limit of the fluid, the latter then being blown out in a watch-glass. Equal amounts of 0·8 per cent. salt solution can be measured out with the marked tube and added till the fluid has the necessary volume. Thorough mixture is effected by drawing up the diluted serum in the quill tubing and blowing out again, this being repeated several times. Further dilutions can be made by the graduated pipettes.[2]

Method of using Pipettes.—A mouthpiece of glass quill tube is attached to the pipette by indiarubber tubing. To obviate the danger of sucking up living cultures into the mouth a small piece of cotton wool may be packed loosely into one end of the indiarubber tube. The mouthpiece is held in one corner of the mouth between the teeth, and the junction between the glass stem of the pipette and the attached indiarubber tube is held between the thumb and first and second fingers of the right hand. In order to draw up a

[1] These are supplied by instrument makers, such as Hawksley & Sons, London.

[2] For Wright's methods of measurement, reference should be made to his work on the *Technique of the Teat and Capillary Glass Tube*, London, 1921.

measured amount, say 0·05 c.c., into the fine pipette—(1) draw up fluid to slightly above the 0·05 mark, then apply the tip of the tongue to the orifice of the mouthpiece, so as to close it, and continue to keep the tongue in this position until operation (4) is completed; (2) withdraw the nozzle of the pipette from the fluid, and bring it against the side of the vessel above the level of the fluid; (3) while maintaining the nozzle continuously against the vessel wall make slight pressure on the rubber with the fingers and thumb of the right hand until the level of the fluid in the pipette reaches the 0·05 mark; then do not make any further pressure; (4) then insert the pipette into the vessel into which the fluid is to be delivered (if the amount to be delivered is very small, *e.g.* 0·01 c.c., pass the nozzle down to within a short distance of the fluid in the tube) and (5) blow out vigorously—while blowing, bring the nozzle into contact with the side of the tube above the level of the contents. (The object of bringing the nozzle against the vessel wall in (3) and (5) is to ensure that the fluid shall drain away from the pipette and not remain at the nozzle as a drop, which would cause a serious error in the measurement of the amount delivered.)

The Drop Method.—Another method of measuring fluids depends on the use of dropping pipettes. When one pipette is used throughout, the procedure is simple and it is accurate when fluids such as serum and saline are concerned. The pipette, which consists of a length of quill tube drawn out to a capillary end, is actuated by an indiarubber teat; the pipette must always be held vertically and the drops must be delivered slowly (at the rate of about 1 drop per second). Before passing from one reagent to another the pipette must be well washed out and either dried with alcohol and ether or rinsed out with the reagent to be measured by taking up and rejecting the latter several times. (When pipettes have to be interchanged, Donald's methods of standardising should be used.)

Agglutination.—By *agglutination* is meant the aggregation into clumps of uniformly disposed bacteria in a fluid; by *sedimentation* the formation of a deposit composed of such clumps when the fluid is allowed to stand. Sedimentation is thus the naked-eye evidence of agglutination. The blood serum may acquire this clumping power towards a particular organism under certain conditions—these being chiefly met with when the individual is suffering from the disease produced by the organism, or has recovered from it, or artificially as the result of injections of the organism. The nature of this property will be discussed later. Here we shall only give the technique by which the presence or absence of the property may be tested. There are two chief methods, a microscopic and a naked-eye, corresponding to the effects mentioned above. In both, the essential process is the bringing of the diluted serum into contact with the bacteria uniformly disposed in a fluid. In the former this is done on a glass slide, and the result is watched under the microscope; the occurrence of the phenomenon is shown by the aggregation of the

bacteria into clumps, and if the organism is motile this change is preceded or accompanied by more or less complete loss of motility. In the latter method the mixture is placed in an upright thin glass tube ; sedimentation is shown by the formation within a given time (say from two hours at 55° C. to twenty-four hours at room temperature) of a somewhat flocculent layer at the bottom, the fluid above being clear. Two points should be attended to : (*a*) controls should always be made with normal serum and with the bacterial emulsion alone, and (*b*) the serum to be tested should never be brought in the undiluted condition into contact with the bacteria. The stages of procedure are the following :

Blood having been obtained and the serum separated (for methods see pp. 133, 145)—

1. The serum may be diluted (*a*) by means of a graduated pipette or by the drop method. In this way successive dilutions can be rapidly made in $3 \times \frac{1}{2}$ inch test-tubes. In general, a series of doubling dilutions is suitable, *e g* 1 : 5, 1 : 10, 1 : 20, etc., though, of course, any relation between the successive dilutions may be selected. This is the best method (*b*) By means of a capillary pipette with a mark on the tube. The serum is drawn up to the mark and then blown out into a glass capsule ; equal quantities of diluting fluid (usually 0·85 per cent. NaCl solution) are successively measured in the same way, and added till the requisite dilution is obtained. (*c*) By means of a platinum needle with a loop at the end (Delépine's method). A loopful of serum is placed on a slide, and the desired number of similar loopfuls of diluent are separately placed around on the slide. The drops are then mixed.

2. The bacteria to be tested should be taken from young, well-grown cultures, preferably not more than twenty-four hours old, incubated at 37° C. They may be used either as a bouillon culture or as an emulsion made by adding a small portion of an agar culture to bouillon or 0·85 per cent. solution of sodium chloride. In the latter case successive small amounts of saline are added to the mass of bacteria, and suspension is aided by gently rubbing the culture with a looped needle. When a thick turbidity is thus obtained, the suspension is allowed to stand for half an hour at 37° C. to let gross particles settle out, and then the organisms should be uniformly mixed with the rest of the fluid. The bacterial emulsion ought to be distinctly turbid. In the case of organisms of the enteric group, 3 to 5 c.c. fluid are used to suspend one agar culture, so as to give a density of 1000–2000 million organisms per c.c. as judged by the opacity (see p. 141). If living organisms are used, precautions must be taken against accidental infection. For most purposes suspensions killed by exposure at 56° C. for half an hour are suitable, or killing may be effected by using saline to which 1 : 1000 formalin has been added.

3. To test *microscopically*, mix equal quantities of the diluted serum and the bacterial emulsion on a glass slide, cover with a cover-glass, and examine under the microscope. The form of glass slide used for hang-drop cultures (Fig. 70) will be found very suitable.

The ultimate dilution of the serum will, of course, be double the original dilution.

To observe *sedimentation*, mix equal parts of diluted serum and of bacterial emulsion, and place in an agglutination tube or a narrow test-tube ($\frac{1}{8}$ inch diameter). Keep in the upright position for twenty-four hours at room temperature, at 37° C. for four hours, or at 55° C. in a water bath for two hours (the convection currents set up when the tubes are only partially immersed hastens the appearance of flocculation). It is important to observe not merely the fact that agglutination occurs, but also the weakest concentration of the serum with which the reaction can be obtained. Thus if the highest dilution of the serum which produces agglutination is 1 : 8000, this is expressed by saying that 8000 is the " titre " of the serum. The details of the procedure are as follows in the case of testing a patient's serum with B. typhosus. A series of small ($3 \times \frac{1}{2}$ inch) test-tubes, *e.g.* six, is set up. Into each of the tubes with the exception of No. 1, 0·4 c.c. saline is measured. Then to 0·1 c.c. serum there is added 1·4 c.c. saline, thus making a 1 : 15 dilution ; of this dilution 0·4 c.c. is added to tubes Nos. 1 and 2, and the contents of the latter are mixed by drawing up into the pipette several times. From No. 2, 0·4 c.c. is transferred to No. 3, and, after mixing, 0·4 c.c. from the latter is transferred to No. 4 and so on, up to tube 5, 0·4 c.c. from No. 5 being rejected. The result is that tubes Nos. 1 to 5 each contains 0·4 c.c. of the following dilutions of serum—1 : 15, 1 : 30, 1 : 60, 1 : 120, 1 : 240 ; tube No. 6 contains no serum. Now 0·4 c.c. of bacillary suspension is added to each tube and the contents are mixed and transferred by means of a capillary pipette to the agglutination tubes, beginning with No. 6 and proceeding backward in order to No. 1. Thus the bacteria are exposed to the following concentrations of serum—1 : 30, 1 : 60, 1 : 120, 1 : 240, 1 : 480. The results are best read with the naked eye by holding the tubes against a dark background, with a bright light in front of the observer. The naked-eye method of testing for agglutination is much preferable to the microscopic, as the results with the former are more accurate.

Flocculation on a slide is a useful method for preliminary observations, *e.g.* when testing suspicious colonies from plate cultures of fæces in order to determine which should be submitted to further examination. Drops of a low dilution of agglutinating serum (*e.g.* 1: 100 of an antiserum with a titre of 5000) are placed on a slide and a loopful of organisms from each colony is rubbed up with the needle in successive drops, so as to yield a very dense turbidity ; then the slide is rocked vigorously for a minute or so. If flocculation occurs, obvious granularity to the naked eye develops.

Standard Agglutinable Cultures.—Since different cultures of the same organisms (and even different subcultures of a single strain) often differ in their agglutinability by the same antiserum, Dreyer has devised a method of standardising such cultures. The organism (*e.g.* B. typhosus) is grown for twenty-four hours at 37° C. in ordinary veal peptone bouillon in an Erlenmeyer flask. At the end of this time the flask is shaken, and there is added to it 0·1 per cent. of commercial formalin ; it is again shaken and placed at once in a cool chamber at about 2° C. in the dark. The shaking is repeated at intervals for four to five days, the flask always being replaced in the cold chamber. At the end of three or four days the culture

will be found to be sterile and will keep practically indefinitely. Such killed cultures are very suitable for sedimentation tests. When a new specimen of culture is to be employed, it is tested for agglutination with varying dilutions of an antiserum, so as to determine the titre of the latter. Simultaneously, the titre of the same antiserum is determined when used along with the standard culture. If the two titres differ, then the latter divided by the former yields a factor which must be employed to multiply the titres of all sera tested with the new culture in order to make them comparable with results obtained with the standard culture. In this way successive batches of agglutinable culture are compared and the factor for each is determined.[1]

In testing a patient's serum Dreyer recommends preparing by the drop method a series of dilutions of 1 : 25, 1 : 50, 1 : 125, 1 : 250, and a control without serum; the mixtures are kept at 50°–55° C. for two hours in the case of the enteric organisms (four hours in the case of dysentery bacilli) and the results are read after the tubes have stood for fifteen minutes further at room temperature. "Standard agglutination" represents flocculi just visible to the naked eye.[2] (For further details of this method reference should be made to the Medical Research Council's Special Report Series, No. 51.)

The content of a given serum in "agglutinin units" is obtained by dividing its titre by a factor which is compounded of the figure obtained as described above along with an arbitrary number so chosen that in the case of a normal serum the number of units will not exceed ten. Hence a result of over ten agglutinin units is to be taken as a positive result, *i.e.* as indicating a content of the serum in agglutinin which is higher than normal.

The Absorption Method of testing Agglutinins.—This method is applied when several agglutinins acting on allied organisms are present in a serum. The principle is to remove all the agglutinins acting on one organism, and to study the properties of those which remain. In practice, the method consists in adding to a suitable dilution of the serum (*e.g.* a serum whose titre was 5000 would be diluted ten times) an equal volume of a thick emulsion of the bacterium (the organisms of one agar slope being used for 2 c.c. of diluted serum), allowing the mixture to stand at 37° C. for two or three hours, and then separating the bacteria with the centrifuge. The supernatant clear fluid is now pipetted off, and its agglutinating properties studied on the other members of the bacterial group and also, by way of control, on the organism used for absorbing it, in order to make sure that all agglutinins for the latter have been removed. The object of the method is to determine which member of a bacterial group is causally related to the condition

[1] These agglutinable cultures are supplied by the Pathology Department, University of Oxford.

[2] Control tubes showing standard agglutination for enteric and dysentery bacilli are obtainable from the Oxford Laboratories.

from which the serum is obtained, and examples of its application for this purpose will be found in the chapter on Typhoid Fever (p. 439). Here the principle is that, when an unknown strain belonging to such a bacterial group is under investigation, if its capacities for absorbing agglutinins from a serum are the same as those of an already recognised strain, then the two are probably identical. On the other hand, an allied strain to the organism by which the agglutinin has been produced will absorb only part of the agglutinin.

Method of obtaining Agglutinating Sera.—For the identification of organisms by their agglutination reactions artificial antisera of high titre must always be employed (human sera from cases of natural infection should never be used). Rabbits are in general most suited for developing specific antisera; old animals ought to be avoided. Cultures killed by heat are generally effective, as low a temperature as possible being employed, *e.g* half to one hour at 56° C. Intravenous injection is as a rule to be preferred, a small dose being given at first (1 : 50 of a killed twenty-four-hour agar-slope culture suspended in saline), followed by gradually increasing doses (each being double the preceding one) at intervals of seven to ten days. During the course of immunisation the animal should be weighed frequently. Loss of weight suggests that the dosage is too high; if the animal's health is seriously upset, powerful antisera will probably not be obtained. But it must be remembered that some animals respond to the injections by more active production of antibodies than others. In the case of highly toxic organisms, such as Shiga's dysentery bacilli, a smaller initial dose is used, *e.g.* 1 : 100 of a culture, or their toxicity may be diminished by heating at 80° C. for an hour. Seven to ten days after the second or third dose a specimen of blood may be withdrawn from the ear vein and tested. If the titre is high (2000 or more) the animal may be bled; 20 c.c. of blood may be obtained from the ear vein, and this may be repeated on several successive days provided the animal is supplied with abundance of water to drink. Or the animal may be anæsthetised with ether and bled from the carotid artery. The blood is received into a sterile tube which has been rinsed out with saline to ensure retraction of the clot. The serum which has separated from the clot on standing for twenty-four hours in a cool place is stored in lengths of quill tubing drawn out at the ends, which are sealed in the Bunsen flame. It may be preserved by heating for half an hour at 56° C. several times, or by the addition of 0·1 c.c. of 5 per cent. solution of phenol in 0·85 per cent. NaCl solution to each c.c. of serum, and kept in the ice-chest; or the serum, without any treatment or addition, may be kept frozen.

Opsonic Methods

Method of measuring the Phagocytic Capacity of the Leucocytes.—This was first done by Leishman by a very simple method, as follows:

Equal quantities of blood and of a fine emulsion of the bacterium to be tested are mixed together, a small drop of the mixture is placed on a glass slide and covered with a cover-glass ; the preparation is placed in the incubator at 37° C. for fifteen minutes. The cover-glass is then slipped off and the film on the slide stained by Leishman's method. A control preparation can be made with normal blood in the same way, and the two films are stained as one. The number of bacteria present in, say, fifty polymorpho-nuclear cells successively examined is determined, and an average struck.

By this method Leishman showed that in cases of staphylococcus infection the average number of bacteria taken up was less than in a control in which the same bacterial emulsion was exposed to the blood of a healthy individual. Wright subsequently showed that phagocytosis depended upon certain substances in the serum to which he gave the name *opsonins* (p. 201), and elaborated the following method by which its degree could be estimated :

(1) *Preparation of Bacterial Emulsion.*—In the case of organisms such as the pyogenic cocci, some of a twenty-four-hour living culture on a sloped agar tube is taken and rubbed up with 0·85 per cent. saline so as to obtain an emulsion consisting of single bacterial cells. With certain organisms, *e.g.* streptococci in chains, a good deal of trituration may be necessary, and often centrifuging must be practised, for the removal of clumps. Only by experience can a knowledge be gained of the amount of culture to be used in the first instance, but the resultant emulsion usually should exhibit only the merest trace of cloudiness to the naked eye. If too strong an emulsion be used, the leucocytes may take up so many organisms that these cannot be accurately enumerated. When intensely pathogenic organisms are used—*e g.* B. pestis, M. melitensis —Wright recommends that the culture should be first killed by emulsifying in 40 per cent. formalin. The latter is then removed by centrifuging and the deposit washed with saline. In the case of the tubercle bacillus, Wright directs that a seven- to ten-day culture in glycerine broth should be sterilised by heat, collected on a filter, washed with salt solution, and dried. Ten milligrams of the dry culture should be powdered in a small agate mortar, a drop of 1 per cent saline added, and the sticky paste triturated for about five minutes ; further saline is added drop by drop till a thick emulsion is obtained of the bulk of about 1 c.c This is centrifuged and the supernatant suspension pipetted off and diluted to the necessary degree.

(2) *Preparation of Leucocytes.*—Here the observer uses his own blood cells. A 1·5 per cent. solution of sodium citrate in 0·85 per cent. sodium chloride is prepared. This is placed in a centrifuge tube, which is filled nearly to the brim. A handkerchief being bound round the finger, this is now pricked, and the blood allowed to flow directly into the fluid, to the bottom of which it sinks. The tube ought to be inverted between the addition of every few drops of blood, so as to bring the blood in contact with the citrate and prevent coagulation. The equivalent of about 10 to 20 drops of blood should be obtained. The diluted blood is then centrifuged,

and when the corpuscles are separated the supernatant fluid is removed, fresh saline is substituted, and the centrifuging repeated. A second washing with saline is practised, the supernatant fluid removed, and the greyish surface layer of blood, which is rich in leucocytes, removed by a fine pipette. The leucocytes may be thoroughly mixed by drawing up in a fine pipette and blowing out again, this being repeated several times.

(3) *Preparation of the Sera.*—Each sample of serum is prepared by the methods described on p 147. In every case serum from a normal individual should be prepared as a control

The emulsion, corpuscles, and serum being thus prepared, an equal quantity of each is taken by a small capillary pipette, and a thorough mixture is made in the usual way. A small portion of the mixture is taken up in a capillary tube, and its ends are sealed by heat, care being taken that the contents are not over-heated. The tube is then placed in the incubator at 37° C. for fifteen minutes. At the end of this time a drop of the mixture is placed on a slide, and a film preparation is made, this in the case of ordinary bacteria being stained by Leishman's method. With tubercle bacilli the following is the procedure : The film is fixed, washed thoroughly, stained with carbol-fuchsin as usual, decolorised with 2·5 per cent. sulphuric acid, cleared with 4 per cent. acetic acid, washed with water and counter-stained with watery solution of methylene-blue (to which ½ per cent. sodium carbonate may be added), and dried.

The two preparations are now examined microscopically with a movable stage, the number of bacteria in the protoplasm of at least a hundred polymorphonuclear leucocytes is counted, and an average per leucocyte struck ; the proportion which this average in the case of the abnormal serum bears to the average in the preparation in which the healthy serum was used, constitutes the *opsonic index*—that of healthy serum being reckoned as unity.

In the case of such organisms as those of the coli-typhoid group and cholera, which are susceptible to bacteriolytic influences in the serum, it may be necessary to heat the sera of the patient and observer for half an hour at 55° C. This destroys any complement present and prevents bacteriolysis occurring. In the case of the B. typhosus the virulence of the strain employed has been shown to be an important factor.

Several modifications of Wright's technique have been suggested. For example, Simon compares not the numbers of bacteria ingested, but the percentages of cells containing bacteria and those not containing bacteria. This he calls the " percentage index," and he states that the figure thus obtained corresponds very closely to the ordinary opsonic index ; he claims that the method eliminates some of the errors which may arise in the use of the ordinary technique if only a relatively small number of phagocyting cells, such as fifty, be examined.

Bactericidal Methods—Fixation of Complement

The Estimation of the Bactericidal Action of Serum.—This may be carried out by various methods, of which those of Neisser and Wechsberg and of Wright may be given as examples.

In the former, the effects of varying amounts of serum on the same amounts of bacteria are observed by means of plate cultures; in the latter, the number of bacteria which can be completely killed off by a given quantity of serum is ascertained. In carrying out experiments of this kind it is convenient to have a number of small test-tubes sterilised and plugged with cotton-wool. We can then make any required dilution of a young bacterial culture in bouillon as follows: To each of a number of tubes we add 0·9 c.c. of 0·85 per cent. solution of sodium chloride. To the first tube (*a*) we add 0·1 c.c. of the bacterial culture, and thoroughly shake up the mixture; to the second (*b*) we add 0·1 c.c. of the contents of (*a*), and shake up; to the third tube (*c*) we add 0·1 c.c. of the contents of (*b*), and so on. It is thus evident that 0·1 c.c. of the contents of (*a*) will correspond to 0·01 c.c., and 0·1 c.c. of (*b*) to 0·001 c.c. of the original culture; any required fraction can thus be readily obtained. In the making of the mixtures it is essential that none of the bacteria shall escape the action of the serum, *e.g.* by remaining on a part of the mixing vessel with which the serum does not come in contact.

(*a*) *Method of Neisser and Wechsberg.*—A series of small plugged sterile tubes is taken, and to each we add 0·5 c.c. of 0·85 per cent. sodium chloride solution, and a given quantity, say $\frac{1}{50}$ c c., of a young bouillon culture to be tested. To the several tubes in series we then add varying amounts of the fresh serum whose action is to be observed, *e.g.* 0·2 c c., 0·1 c c, 0·05 c.c., 0·025 c.c, etc. The contents of each tube are then made up to 1 c.c. with salt solution, and a few drops of sterile bouillon are added to each tube. The tubes are then well shaken and placed in the incubator at 37° C. for three hours, to allow the serum to act (Of course several series of such tubes may be prepared and placed in the incubator for varying periods of time; we can thus observe when the bactericidal effect reaches the maximum.) At the end of the given period of time a small quantity, say 0·05 c.c., of the contents of each tube is added to a tube of melted agar (cooled to about 45° C.); each agar tube is then shaken, and the contents are poured out into a sterile Petri capsule. The other tubes are similarly treated, and the Petri capsules are placed in the incubator for a suitable period of time. The number of colonies in each can then be noted. Of course gelatin can be substituted for the agar in the plates if desired.

(*b*) *Wright's Method.*—A twenty-four hours' bouillon culture is used, and various dilutions with sterile bouillon are made according to the method described above; thus 5-, 10-, 20-, 50,- 100-, 1000-, etc., fold dilutions may be prepared. A small quantity, say 1 c.mm., of the fresh serum to be tested is mixed with an equal amount of the bacterial culture, and the mixture is placed in a small capillary tube which is sealed at the ends; similar mixtures of equal parts of serum and of each of the dilutions of culture are prepared and treated in the same way. The tubes are then placed in the incubator

for eighteen to twenty-four hours at 37° C., and at the end of that time the contents of each are tested as regards sterility by means of cultures. In this way the greatest dilution in which the bacteria are completely killed off is ascertained. The number of bacteria in the original culture per c.mm. can be counted by the method given on p. 76, and thus the total number of bacteria killed off by the quantity of serum used can be readily calculated.

As will afterwards be described in greater detail (*see* Chapter on Immunity), when an animal is immunised against a particular bacterium the bactericidal action of its serum may be greatly increased, and this depends on the development of a particular substance called an immune-body, which is comparatively thermostable and is not destroyed at 55° C. To analyse the bactericidal properties of such a serum, it should in the first place be heated in order to destroy the normal complement. Then to each of a series of sterile tubes we add (*a*) a quantity of normal unheated serum insufficient of itself to destroy the bacteria, (*b*) a given amount of the bacterial culture, and (*c*) varying amounts of the heated immune-serum—0·1, 0·01, 0·001, etc., c.c. In this way we can find the quantity of the immune-serum which gives the maximum bactericidal action.

In some cases, however, when an animal is immunised against a given bacterium, or when a patient is infected with the organism, the serum may not have increased bactericidal action, but nevertheless contains an immune-body which leads to the absorption or fixation of complement. In other words, the immune-body is a substance which, along with the corresponding or homologous bacterium, binds complement (p. 197). In order, however, to explain the methods by which the fixation of complement may be demonstrated, we must first of all give some facts with regard to hæmolytic sera.

Methods of Hæmolytic Tests.—A hæmolytic serum is usually prepared by injecting the red corpuscles of an animal into the peritoneum of an animal of different species—the corpuscles of the ox or sheep are most frequently used, and the rabbit is the most suitable animal for injection. The corpuscles ought to be completely freed from serum by repeatedly washing them in sterile salt solution, and centrifuging. An intraperitoneal injection of the corpuscles of 5 c.c. of, say, ox's blood, followed by two injections, each of 10 c.c., at intervals of eight days, will usually give an active serum. About a week after the last injection a specimen of blood should be taken from the ear and its titre estimated. If this is satisfactory, the animal should be anæsthetised and bled from the carotid. The serum which separates (after centrifuging to remove any suspended red corpuscles) may be collected in suitable lengths of quill glass-tubing drawn out at the ends, which are afterwards sealed in the flame. To ensure sterility when the serum is to be kept some time, it is advisable to heat it for an hour at 55° C. on three

successive days; we have always found that serum treated in this way remains sterile. If is, of course, devoid of complement. The test amount of corpuscles is usually 1 c.c. of a 5 per cent. suspension of corpuscles in 0·85 per cent. sodium chloride solution; that is, the corpuscles of 5 c.c. blood are completely freed of serum by repeatedly washing in salt solution, and then salt solution is added to make up 100 c.c. In any investigation it is necessary to ascertain the minimum hæmolytic dose (M.H.D.) of the immune-body and of the complement to be used. (It is to be noted that as complement does not increase during immunisation, the hæmolytic dose of the fresh serum will come far short of representing the amount of immune-body present.) In testing the *dose of immune-body,* the fresh serum to be used as complement must be devoid of hæmolytic action (in the present instance rabbit's serum will be found suitable), and more than sufficient to produce lysis with immune-body is added to each of a series of tubes. Instead of rabbit's serum it is preferable to use guinea-pig's serum as the source of complement, since it is much more active. The latter often contains some natural immune-body for ox's red corpuscles, which may be removed by the following procedure. Equal volumes of guinea-pig's serum and of sharply centrifuged washed ox blood sediment in separate tubes are chilled by immersion for five minutes in chopped ice, then they are mixed and the mixture is kept on ice for an hour. The treated serum is recovered by centrifuging and is pipetted off; it is now devoid of any lytic action by itself, but contains the complement; 0·1 c.c. of this treated serum is added to each tube in estimating the dose of immune-body. Varying amounts of immune-body (which should be diluted with saline in order to permit of accurate measurement) are added to the tubes, the contents are shaken, made up to 1·5 c.c., and incubated for two hours. The amount of lysis is then noted, and the tubes are placed in a cool chamber till next morning, when a final reading is taken. The smallest amount of immune-body which gives complete lysis is, of course, the M.H.D.: sometimes this may be as low as 0·001 c.c. for the test amount of corpuscles. When further observations are to be continued on the same day, the reading after incubation must be taken as the working standard. To estimate *the M.H.D. of complement,* proceed in a corresponding manner; to each of a series of tubes add several (at least five) doses of immune-body, and then to the several tubes different amounts of complement. Nearly maximal lysis is obtained after one hour's incubation. The amount of complement necessary for lysis varies somewhat

according to the amount of immune-body used, being smaller with several doses of the latter than with a single dose ; in estimations of the dose of complement, it is accordingly advisable to use the optimum amount of immune-body, in the present instance about five hæmolytic doses. The activity of a serum as complement varies considerably, and each sample must be separately tested.[1] Corpuscles treated with sufficient immune-body to produce complete lysis on the addition of complement are usually spoken of as *sensitised corpuscles*. The above will serve as an indication of the fundamental methods ; for further details, special papers on the subject must be consulted.

The Removal of Blood-Samples from Rabbits.—In such work as that just described, it is often convenient to watch the progress of an immunisation procedure by removing a sample of blood without the animal being killed. With proper care any amount of blood up to one-third of that contained in the body can be removed from the ear vein of a rabbit. The animal, which must not be flurried, is placed on a bench, and its body kept warm by being covered with a cloth. The root of the ear should be shaved over the marginal vein, the hairs on the edge of the ear should also be clipped short. It is best to have the ear dry, as the evaporation of a fluid causes contraction of the vessels. In a great deal of hæmolytic work absolute sterility of the sample is not necessary, so that washing the ear is not required. When sterile blood is desired, the precautions detailed on p. 53 may be applied. A frosted incandescent electric lamp, such as is used for microscopic illumination, is placed lighted an inch or two from the ear. The left hand of the operator should cover the animal's head in front of the ears, the thumb and index-finger being left free to compress the vein at the foot of the ear. In this way not only is the animal's eye protected from the glare of the lamp, but the distance of the latter from the ear can be regulated so as to keep it at what to the operator's hand is a pleasant warmth. In a minute or two the ear vessels will dilate, and the vein, being compressed at the root, a lateral opening is made with a bayonet-pointed surgical needle (the triangular-pointed needles supplied with the Gowers-Haldane hæmoglobinometer are also very suitable), and the blood allowed to drop into a sterile test-tube. Usually waves of contraction of the ear vessels will be observed to occur, the passing off of which must be waited for, and from time to time the clot must be gently squeezed out of the opening in the vein with the flat side of the needle, or it may be necessary slightly to enlarge the opening. The blood should be allowed to clot completely, and then, by means of a sterile platinum

[1] Complement rapidly (often within twenty-four hours) loses its strength when kept at room temperature. It can, however, be preserved for a considerable time at or near its original strength if it be kept frozen. Even if this be done, however, the strength of the complementary serum must be titrated at the commencement of every experiment in which it is employed. Commonly the M.H.D. of fresh guinea-pig's complement for 1 c.c. ox or sheep corpuscle suspension is 0·01 or 0·015 c.c.

needle, the clot should be loosened from the sides of the tube in order that it may freely contract. The tube should be placed in the ice-chest till the following morning, when the serum can be pipetted off with a sterile pipette.

Daily samples can thus be obtained from an animal. If care be taken not to make ragged openings in the vein, often the simple removal of the previous scab will be followed by a free blood flow.

This method can be applied in guinea-pigs, provided these be of fair size. Here successive samples of 2 c.c can be obtained from the ear veins.

Fixation of Complement or Complement Deviation (Reaction of Bordet and Gengou).—From the facts given above it follows that sensitised corpuscles, *i.e.* corpuscles treated with immune-body, may be made to serve as an indicator for the presence of complement. If an antibacterial immune-body is present in a serum heated at 55° C., the serum when added to the corresponding bacterium leads to the fixation of complement, and thus prevents hæmolysis when sensitised corpuscles are added. If we represent the bacteria, or rather the receptors in the bacteria, by X, the immune-body by anti-X, and the complement by C (normal serum, say, of a guinea-pig), we may represent the method of experiment by the following scheme :

$$X+\text{anti-}X+C \;\vdots\; +\text{sensitised corpuscles} \;\vdots$$

(The vertical dotted line represents a period of incubation for one and a half hours at 37° C.)

If lysis of the sensitised corpuscles does not occur after incubation at 37° C., then the complement has been fixed and an immune-body has been shown to be present, provided that a suitable control shows that the bacteria alone, without immune-body, do not fix sufficient complement to interfere with lysis.

This method has now been extensively used for demonstrating the presence of immune-bodies in the blood of patients suffering from a particular bacterial infection. It has also been applied to determine whether a suspected bacterium is really the cause of a disease, for if the bacterium gives with the serum of the patient fixation of complement, then there is a strong presumption that it is the infective agent (*vide* Immunity). The method as carried out is practically the same as that of the Wassermann reaction (see below) except that the antigen consists of broth cultures or suspensions in saline of slope cultures of the organisms, which have usually been killed by heating. The optimum amount of organisms to be employed

must be ascertained in every instance, but the test amount of bacterial suspension should not by itself inhibit more than one or two doses of complement. It is essential that antisera which are to be tested in this way should not have been obtained from animals injected with cultures grown on foreign blood or serum, since antibodies generated by the latter may lead to fallacies. Complement fixation may be said to occur when the mixture of antigen and serum inhibits more complement than the sum of the amounts inhibited by each of these reagents separately; but the phenomenon is clearly present when the mixture of antigen with the serum of an individual infected or inoculated with the organism in question inhibits complement to a greater degree than does a similar mixture containing serum from an uninfected individual.

The Serum Diagnosis of Syphilis (Wassermann Reaction : Flocculation Reaction).—Wassermann, Neisser, and Bruck, proceeding in accordance with the facts established with regard to the fixation of complement, tested whether a similar phenomenon might not be obtained in the case of syphilis. For this purpose they mixed together a watery extract of syphilitic liver, rich in spirochætes (antigen), and serum from a syphilitic case (supposed to contain anti-substances), and found that a relatively large amount of complement was fixed. On the other hand, when the serum from a non-syphilitic case was substituted for the syphilitic serum, little or no fixation of complement occurred. The result was thus in accordance with expectations on theoretical grounds. Marie and Levaditi, however, found that an extract of normal guinea-pig's liver along with syphilitic serum fixed complement, *i.e.* acted as antigen. Subsequent observations (Porges and Meier and others) showed that alcoholic extracts of normal as well as syphilitic tissues are also more or less efficient, and the property resides mainly in the fraction of the extract which is soluble in alcohol and ether but insoluble in acetone (lecithin). The addition of cholesterol to lecithin enhances greatly its activity (Browning, Cruickshank, and Mackenzie). Although abundant observations have established the validity of the test as a means of diagnosis, the reaction which led to its discovery is no longer sufficient to explain it.

In order to carry out the test, we require (*a*) serum from the suspected case, (*b*) an alcoholic tissue extract (that of human or ox heart, along with cholesterol, being most widely used), (*c*) the fresh serum of a guinea-pig to act as complement, and (*d*) sensitised ox corpuscles, *i.e.* a 5 per cent. suspension of washed ox corpuscles to which five doses of immune-body have been

added (p. 132)—a similar suspension of sheep corpuscles is often used.

The following may be given as an example of the method as formerly used :

Add to a small test-tube containing 0·5 c.c. of 0·8 per cent. sodium chloride solution : (*a*) 0·05 c.c. of serum from the suspected case (heated for an hour at 55° C. to destroy the complement, etc), (*b*) 0·1 c.c. of an extract of guinea-pig's or ox's liver, and (*c*) a certain amount, usually 0·1 c c. of guinea-pig fresh serum, to act as complement. Place in incubator at 37° C. for an hour and a half. At the end of that time add 1 c.c of sensitised ox corpuscles and place in the incubator for another hour. If at the end of the hour the corpuscles are not lysed the complement has been fixed in the first stage—the result is *positive* as regards the presence of syphilis ; if lysis has occurred the result is *negative*. Controls were used to test effect on complement of (*a*) suspected serum alone and (*b*) antigen alone.

It is to be noted, however, that the substance in the syphilitic serum which leads to the fixation of complement varies greatly in amount in different cases, and in the same case at different times, especially under the influence of anti-syphilitic treatment with salvarsan. Accordingly, it is not possible to state absolutely the quantity of complement which must be fixed in order to give a positive result. Manifestly there will be cases where the amount fixed is just under any standard adopted, and these, which are to be regarded as suspicious or doubtful, will be missed with a one-tube method. Moreover, the amount of complement, as estimated by the hæmolytic dose, varies considerably in different samples of fresh serum ; also the complement in different specimens of serum varies in its capacity for being fixed (deviability). It is accordingly necessary for satisfactory results to estimate the hæmolytic dose of the guinea-pig's serum, and to prepare a series of tubes, each containing the same amounts of serum and of antigen, but with a different number of doses of complement in each tube. In this way we can find the number of doses of complement fixed in each case. As controls, the effect on doses of complement of the extract alone and of the serum alone should be tested ; also, a known negative and positive serum should be tested at the same time (the latter should be one which gives only a weak reaction).

Quantitative Method.—Three tubes with different doses of complement will be sufficient for routine examination.

To each of these add 0·5 c.c. of *diluted antigen* (*vide infra*) and 0·05 c.c. of the serum to be tested (heated for half an hour at 55° C.).

Add to the three tubes respectively, two, four, and six doses of complement—the dose being that for 0·5 c.c. of sensitised corpuscles

(the mixed sera of several guinea-pigs, which have been bled eighteen hours previously and the blood kept in the ice-chest).

Place the tubes in the incubator for an hour and a half at 37° C.

Then add to each 0·5 c.c. of suspension of ox corpuscles sensitised with five doses of immune-body, and place again in the incubator for an hour and a quarter. Place the tubes aside at the room temperature till the non-lysed corpuscles have sedimented—conveniently till next morning—and then read the results.

Controls should be made in each test as follows : one tube containing the stated amount of antigen along with two doses of complement, and one containing the stated amount of heated serum along with two doses of complement ; to all these 0·5 c c of sensitised corpuscles is added after incubation for an hour and a half. It is also advisable to put up series with a known negative (preferably a pooled specimen of at least six sera) and a weakly positive serum—in routine work it is convenient to keep such sera from previous tests ; they are best preserved by freezing, but if sterile they will keep for a few days in the ice-chest.

Antigen.—Various antigens have been used, but the following gives very satisfactory results :

Human heart muscle from the left ventricle (the cause of death is stated to be immaterial) is freed from visible fat, minced finely, and ground for a minute with absolute alcohol (1 gram of heart to 9 c.c. of alcohol) in a mortar with clean sand. The mixture is shaken in a mechanical shaker for one and a half hours and then filtered. This constitutes heart extract, which keeps at cool room temperature for six months at least.

Preparation of Diluted Antigen —1·5 volumes of heart extract are mixed with 1 volume of 1 per cent. cholesterol in absolute alcohol ; 1 volume of this solution is mixed rapidly with 29 volumes of normal saline. (It is an advantage in the case of each specimen of extract to test various dilutions of it with a known syphilitic serum and find the dilution which gives most fixation, and then to use this dilution in subsequent tests.)

Cerebro-spinal fluid is tested in the same way as serum, except that it is not heated beforehand · 1 volume of the alcoholic antigen is diluted quickly with 29 volumes of the fluid, and 0·5 c.c. of this mixture is measured into each tube. In the control tube, instead of serum and saline, 0·5 c c. of cerebro-spinal fluid is used.

Some observers use the same amount of complement in each tube, but vary the amounts of suspected serum, and in this way an estimate of the deviating power of the serum is obtained, but we consider that the method given is to be preferred.

Reading of Results.—It will usually happen that the test with the antigen alone, with the serum alone, and also that with the mixture of antigen and the negative control serum, show practically complete lysis in the first tube, *i.e.* with two doses of complement. This being the case, the result of the test is decided as follows : (*a*) A serum which shows practically or quite complete lysis with the same amount of complement as causes complete lysis of the negative control serum is *negative.* (*b*) A

serum which requires from one and a half times to twice as much complement as does the negative control to produce a given degree of lysis is *suspicious*—recorded as " ? " or " ± ". (*c*) A serum which fixes more complement than is specified under (*b*), but which shows complete lysis with the highest amount of complement, is a *weak positive*. (*d*) A serum which shows no lysis in any tube is a *strong positive, i.e.* one which fixes upwards of five doses of complement more than the sum of the amounts inhibited by the antigen and the patient's serum separately.

The interpretation must vary somewhat according to circumstances ; for example, a suspicious or weakly positive reaction would be accepted as positive in the case of a syphilitic patient undergoing treatment, but would not be accepted unconditionally as evidence of syphilis in a case in which the diagnosis was in doubt.

Flocculation Reaction.—The occurrence of precipitates when syphilitic sera were mixed with lecithin and other colloids was observed soon after the discovery of the Wassermann reaction, but it is only recently that a reliable form of this test has been devised. The method of Sachs and Georgi will be described.

The sera to be tested, which should be freshly taken, are heated for half an hour at 55° C some hours before use. 2 c.c. each of the alcoholic antigen and of 0·85 per cent. NaCl solution are measured into separate test-tubes ; the saline is then poured rapidly into the alcoholic solution, and the mixture is poured from the one tube to the other several times. It is then allowed to stand for five minutes and 8 c.c. saline are added to it in the same manner as before. This diluted antigen is ready for use after it has stood for from five to fifteen minutes longer at room temperature. For the test two small test-tubes are taken, and into each 0·9 c.c. saline is measured ; then No. 1 receives 0·05 c.c. and No. 2 0·1 c.c. of patient's serum, finally 0·5 c.c antigen is added to each ; a control tube receives 0·9 c.c. saline, 0·1 c c. serum, and (instead of antigen) 0·5 c.c. 16·6 per cent. dilution of absolute alcohol in saline (at the end of the period of incubation this control tube should show no flocculi). The contents of all the tubes are mixed by gentle shaking, and they are incubated at 37° C for eighteen to twenty-four hours. (It has been found that shaking of the mixtures for five to ten minutes in a mechanical shaker before the tubes are placed in the incubator causes flocculation to be more marked and to appear more rapidly in the case of positive sera) The results are read immediately on withdrawal of the tubes from the incubator ; for this purpose they are held between the observer and a bright light, the latter being shaded so that its rays do not pass directly to the observer's eye. In each series of tests the following controls are included : (1) a positive serum, (2) a negative serum, and (3) a control consisting of 0·9 c.c. saline mixed with 0·5 c.c. of the antigen. No. (1) should show flocculation ; Nos. (2) and (3) must appear quite homogeneous. Provided the control series behave as described, any serum which

along with the antigen causes the formation of flocculi visible to the naked eye is said to react positively Positive results may be graded : thus the strongest reaction consists in complete precipitation with perfectly clear supernatant fluid ; the weakest reaction shows fine flocculi just discernible by the naked eye. Positive sera may be compared quantitatively by setting up the test in the case of each serum with a series of dilutions in saline ranging from 1 : 2 to 1 : 64 ; 1 c c. of each dilution is added to 0·5 c c antigen.

This test is less delicate than the Wassermann reaction for the examination of cerebro-spinal fluid

Antigen.—This is prepared by extracting 1 gram of human heart muscle with 5 c.c. alcohol at room temperature for forty-eight hours, shaking occasionally and finally filtering. In the case of each specimen of heart extract it is necessary to ascertain the optimum amount of cholesterol required For this purpose a series of dilutions are prepared consisting of 1 volume of the heart extract with 1, 2, and 3 volumes of absolute alcohol respectively ; then varying amounts of 1 per cent solution of cholesterol in absolute alcohol are added, namely, 0·03, 0 045, 0·06, and 0·075 c c. to 1 c.c. of each of the dilutions of heart extract. The mixtures are diluted with saline as already described, and all are tested with the same negative and positive sera.

The Preparation of Vaccines

In consequence of the work of Sir Almroth Wright, the method of treating bacterial disease by vaccines has been very much developed. The general principle is to inject into the infected individual a suspension of dead bacteria. In certain cases the bacteria are subjected to disintegrating processes before being used, but most frequently the vaccines simply contain killed bacterial cells, and the preparation is comparatively simple.

In the case of organisms such as pyogenic cocci, B. coli, etc., the growth from a young sloped agar culture is suspended in normal saline. A uniform suspension is necessary, and if clumps are present these must be disintegrated with a shaking-machine, or deposited by centrifuging. A sample is withdrawn for the enumeration of the organisms (*vide infra*), and the vaccine is then sterilised by heating in a water bath at 57° C. for one to two hours. With certain staphylococci a longer exposure is advisable, and sometimes in such cases a higher temperature must be employed. It is probable that the lower the temperature at which the contained bacteria are killed the more efficient is the resulting vaccine. The success of the sterilisation must be tested by transferring some of the heated vaccine to an agar tube and incubating for forty-eight hours. Appropriate doses are then, with all aseptic precautions, measured by means of sterile graduated pipettes, and placed in small glass bulbs or

ampoules drawn out to a capillary tube at one end. It is usual to add sufficient ¼ per cent. phenol in sterile saline to make the contents of the ampoule up to about 1 c.c. The ampoules when charged are sealed, and for use the sealed end is broken off, the contents are sucked up into a sterile hypodermic needle, and injected fairly deeply into the loose subcutaneous tissue, *e.g.* of the arm.

In the case of the *typhoid bacillus*, the organism is used of such virulence that a quarter of a twenty-four hours' old sloped agar culture, when administered subcutaneously, will kill a guinea-pig of from 350 to 400 grams. Flasks of bouillon are inoculated with such a culture and kept for forty-two hours at 37° C. The bacteria are then killed by the flask being put into a water bath at 55° C. for twenty minutes ; 0·5 per cent. lysol is added, and the bacteria in the vaccine are counted.

The dosage is adjusted to the standard described in Chapter XV.

Special methods are adopted in preparing the vaccines used in connection with tuberculosis, cholera, plague, etc., and are described in the chapters on these diseases.

Methods of counting the Bacteria in Dead Cultures.—In the making of vaccines it is, as indicated above, advisable to know roughly the total number of bacterial cells, whether dead or living, present in a culture, for the dead as well as the living contain the antigens which may stimulate the production of antibodies. A sufficiently accurate enumeration of the bacteria in a vaccine emulsion can usually be made by counting a suitably diluted sample on a hæmacytometer stage. For this purpose a special cover-glass is supplied, which is ground thin in the centre so that an oil immersion lens can be used. But in many cases a dry lens is sufficient, especially if a small quantity of stain, *e.g.* methylene-blue, is added to the diluent. The diluent ought also to contain some antiseptic, especially when the organisms are motile.

Wright's method consists in making a mixture of blood (whose content in red blood corpuscles is known) with the bacterial culture, and comparing the number of bacteria with the number of corpuscles. The observer first estimates the red cells in his blood ; a capillary pipette with a rubber nipple and with a mark near its capillary extremity is then taken, blood is sucked up to the mark, then an air-bubble, and then an equal volume of the bacterial emulsion diluted according to the empirical estimate the observer forms of its strength. The blood and bacterial emulsion are then thoroughly mixed by being drawn backwards and forwards in the wide part of the pipette, a drop is blown out on to a slide, and a blood film is spread which may be stained by Leishman's method. The bacteria and blood corpuscles are now separately enumerated in a series of fields in different parts of the preparation and the total of each added up. As the number of red corpuscles per c.mm. is known, the number of bacteria can be readily calculated. The results obtained

by the various methods may show considerable differences. In the case of certain bacteria, *e.g.* the members of the coli-typhoid and cholera groups, when an emulsion of these is mixed with whole blood, the serum of the latter may have a bacteriolytic or an agglutinating action on the organisms, which interferes with the counting. In such cases direct enumeration, as above described, should be adopted.

Fairly accurate results, as regards number of organisms, may with practice be obtained by taking a standard opacity of emulsion, representing a known number of organisms, and diluting down with saline the emulsion of organisms to be tested till this opacity is reached. Tubes of the same diameter must of course be used Permanent standards for comparison may be made by preparing suspensions of inorganic substances such as barium sulphate. Brown has elaborated this method [1]

Inoculation of Animals [2]

The animals generally chosen for inoculation are mice, rats, guinea-pigs, rabbits, and pigeons. Care especially must be taken in drawing conclusions from isolated experiments on rabbits, as they are very liable to suffer from intercurrent infections. It must be remembered that different animal species exhibit marked variations in susceptibility to different infections ; therefore in examining material for a particular organism one chooses a susceptible class of animal. On the other hand, in investigating a disease of unknown origin it is essential to inoculate a wide variety of species. The age of the animal also may be an important factor. Animals selected for inoculation should be in good condition, the best evidence of this being that they are gaining in weight ; preferably they should have been kept in the laboratory for some days. Dogs are, as a rule, rather insusceptible to microbic disease, and the larger animals are too expensive for ordinary laboratory purposes. In the case of the mouse and rat the variety must be carefully noted, as there are differences in susceptibility between the wild and tame varieties, and between the white and brown varieties of the latter.

Practically all inoculations are performed by means of a hypodermic syringe. The best variety is that of the " Record " type, furnished with needles preferably of platinum-iridium of various bores. Before use, the syringe should be taken apart, placed in cold water, and sterilised by boiling for five minutes. The

[1] Burroughs Wellcome, London, supply a series of standard opacity tubes for the purpose, along with tables in explanation.

[2] Experiments on animals, of course, cannot, in Britain, be performed without a licence granted by the Home Secretary.

materials used for inocultation are cultures, animal exudations, or the juice of organs. If the bacteria already exist in a fluid there is no difficulty. The syringe is most conveniently filled out of a shallow conical test-glass, which ought previously to have been covered with filter paper and sterilised. If an inoculation is to be made from organisms growing on the surface of a solid medium, either a little ought to be scraped off and shaken up in sterile bouillon or 0·85 per cent. salt solution to make an emulsion, or a little sterile fluid is poured on the growth, and the latter rubbed into it with the looped needle. Gross particles which might obstruct the needle may be removed by letting the fluid stand in the conical glass for a few minutes or by centrifuging at a low speed. If a solid organ is used for inoculation, it ought to be first cut into small pieces and rubbed up in a sterile mortar with sterile sand and a little sterile distilled water; if the emulsion is allowed to stand in a conical glass for a few minutes gross particles soon settle to the foot.

The methods of inoculation generally used are: (1) by scarification of the skin; (2) by subcutaneous injection; (3) by intraperitoneal injection; (4) by intravenous injection; (5) by injections into special regions, such as the anterior chamber of the eye, the cardiac chambers, the substance of the lung, testis, etc. Of these (2) and (3) are most frequently used. When an anæsthetic is to be administered, this is conveniently done by placing the animal, along with a piece of cotton wool or sponge soaked in ether or chloroform, under a bell-jar or inverted glass beaker of suitable size. Another method, *e.g.* as a preliminary to intratesticular inoculation in rabbits, is to inject subcutaneously, three-quarters of an hour beforehand, a dose of 1·5 grams of urethane per kilo. of body weight, and to keep the animal warm.

1. *Scarification.*—Several parallel scratches are made in the skin of the abdomen previously cleansed, just sufficiently deep to draw blood, and the infective material is rubbed in with a platinum eyelet. The disadvantage of this method is that the inoculation is easily contaminated.

2. *Subcutaneous Injection.*—The hair is cut off the part to be inoculated, and the skin purified with alcohol. The skin is then pinched up, and, the needle of the syringe being inserted, the requisite dose is administered. The wound is sealed with collodion.

3. *Intraperitoneal Injection.*—The hair over the lower part of the abdomen is cut, and the skin cleansed. The whole thickness of the abdominal walls is then pinched up between the forefinger and thumb of the left hand, and the needle is plunged through the fold thus formed. If the wall is then relaxed, the point of the needle will be within the abdominal cavity, and the inoculation can thus

be made. In the case of a mouse the animal is held with the head downward and the left leg stretched out backward, then the needle is introduced near the left groin.

4. *Intravenous Injection.*—In the rabbit the vein most usually chosen is one of the auricular veins. The part has the hair removed, the skin is cleansed, and the vein made prominent by pressing on it between the point of inoculation and the heart The needle is then passed obliquely into the vein, and the fluid injected. That it has perforated the vessel will be shown by the escape of a little blood; and that the injection has taken place into the lumen of the vessel will be known by the absence of any swelling in the surrounding subcutaneous tissues

In the rabbit *intraspinal injection* can be made through the space between the seventh lumbar and first sacral vertebrae; the spine of the former lies in a line with the iliac crests. Other operative procedures in special regions of the body are carried out with the usual aseptic precautions.

After inoculation, the animals ought to be kept in cages, which can be thoroughly disinfected subsequently. For this purpose galvanised iron wire cages are the best. It is preferable to have the cages opening from above. Otherwise material which may be infective may be scratched out of the cage by the animal. They can easily be sterilised by boiling in a large fish-kettle. The general condition of the animal is to be observed, and in any experiment in which the animal survives for some time it should be weighed at regular intervals, *e.g.* weekly. The temperature is usually taken *per rectum.* An ordinary clinical thermometer is smeared with vaselin, and the bulb inserted just within the sphincter, where it is allowed to remain for a minute; it is then pushed well into the rectum for five minutes. If this precaution be not adopted a reflex contraction of the vessels may take place, which is likely to vitiate the result by giving too low a reading. The normal rectal temperature of the rabbit is on the average 102·4° F., but marked variations occur, and under 104° F. there is no significant increase.

Collodion Capsules.—These have been used to allow the sojourn of bacteria within the animal body without their coming into contact with the cells of the tissues. Various substances in solution can pass in either direction through the wall by diffusion, but the wall is impermeable alike to bacteria and leucocytes. The following method of preparing such capsules is that of M'Rae modified by Harris: A gelatin capsule, such as is used by veterinary surgeons, is taken, and in one end there is fixed a small piece of thin glass tubing by gently heating the glass and inserting it. The tube becomes fixed when quite cold, and the junction is then painted round with collodion, which is allowed to dry thoroughly. The bore of the tubing is cleared of any obstructing gelatin, and the whole capsule is dipped into a solution of collodion so as to coat it

completely. The collodion is allowed to dry, and the coating is repeated; it is also advisable to strengthen the layer by further painting it at the extremity and at the junction. The interior of the capsule is then filled with water by a fine capillary pipette, and the capsule is placed in hot water in order to liquefy the gelatin, which can be removed from the interior by means of the fine pipette. The sac is filled with bouillon and is placed in a tube of bouillon. It is then sterilised in the autoclave. A small quantity of the bouillon is removed, and the contents are inoculated with the particular bacterium to be studied, or an emulsion of the bacterium is added. The glass tubing is seized in sterile forceps, and is sealed off in a small flame a short distance above the junction The closed sac ought then to be placed in a tube of sterile bouillon to test its impermeability. The result is satisfactory if no growth occurs in the surrounding medium. The sac with its contents can now be transferred to the peritoneal cavity of an animal.

Autopsies on Animals dead or killed after Inoculation.—These should be made as soon as possible after death—in fact, it is preferable to kill the animal when it shows serious signs of illness. It is necessary to use shallow troughs, made of metal or of earthenware. The animal is stretched out and firmly fixed by stout pins to a wooden board which is laid in the trough. The size of the trough will therefore have to vary with the size of the outstretched body of the animal to be examined. Usually the surface of the animal should be soaked in 10 per cent. liquor cresolis saponatus, or in corrosive sublimate (1 : 1000), before it is tied out. This not only to a certain extent disinfects the skin, but, what is more important, prevents hairs which might be infected with pathogenic products from getting into the air of the laboratory. The instruments necessary are scalpels (preferably with metal handles), dissecting forceps, and scissors. They are to be sterilised by boiling for five minutes. Two sets at least ought to be used in an autopsy, and they may be placed, after boiling, on a sterile glass plate covered by a bell-jar. It is also necessary to have a medium-sized hatchet-shaped cautery, or other similar piece of metal. It is well to have sterile capillary pipettes stored in a sterile cylindrical glass vessel, and also some larger sterile glass pipettes. The hair of the abdomen of the animal is removed. If some of the peritoneal fluid is wanted, a band should be cauterised down the linea alba from the sternum to the pubes, and another at right angles to the upper end of this; an incision should be made in the middle of these bands, and the abdominal walls thrown to each side. One or more capillary tubes should then be filled with the fluid collected in the flanks, the fluid being allowed to run up the tube and the point sealed off; or a larger quantity may be taken in

a sterile pipette. If peritoneal fluid be not wanted, then an incision may be made from the episternum to the pubes, and the thorax and abdomen opened in the usual way. The organs ought to be removed with another set of instruments, and it is convenient to place them pending examination in deep sterile Petri's capsules. It is generally advisable to make cultures and film preparations from the heart's blood. To do this, open the pericardium, sear the front of the right ventricle with a cautery, make an incision in the middle of the part seared, and remove some of the blood with a capillary pipette for future examination; or, introducing a looped needle, inoculate tubes and make cover-glass preparations at once. To examine any organ proceed as on p. 149. Fix pieces of the organs for microscopic examination (p. 119). The organs ought not to be touched with the fingers. When the examination is concluded, the body should have corrosive sublimate or carbolic acid solution poured over it, and be forthwith burned. The dissecting trough and all the instruments ought to be boiled for half an hour. The amount of precaution to be taken will, of course, depend on the character of the bacterium under investigation, but as a general rule every care should be used.

Methods of Obtaining Pathological Material for Examination

All specimens intended for bacteriological examination should be taken with such precautions as will reduce to a minimum the chances of contamination from external sources. At the same time admixture with antiseptics must be avoided. Specimens must be received directly into sterile vessels which are at once closed with suitable sterile stoppers. In most cases the examination (making of cultures, films, animal inoculation) should be carried out soon after withdrawal of the material from the body, as many pathogenic organisms soon tend to die or, when present in mixtures, to be overgrown.

Blood for Cultivation, obtaining Serum, etc., from the human subject should be taken from a vein. The skin of the patient's arm at the bend of the elbow is cleansed with sterile gauze swabs soaked in alcohol; then a tourniquet, *e.g.* several turns of stout bandage or indiarubber Bunsen tubing, is applied round the upper arm about the middle of the biceps so as to render the veins turgid, but not to obliterate the radial pulse. Congestion of the veins is aided by the patient opening and closing his hand vigorously; in difficult cases the arm may be immersed in a basin of warm water. (In stout subjects the vein may be palpable to the finger as an elastic cord

when it cannot be seen.) The skin at the elbow is rendered tense from behind by the operator's left hand; the syringe, with needle attached, is held in the right hand and almost parallel with the patient's arm; then the needle, with the eye turned away from the skin, is inserted into a prominent vein. The median basilic is usually the vein which is most easily entered, the needle being pointed either in the direction of or against the blood stream. From 5 to 10 c.c. of blood are drawn up into the syringe, the operator's left hand holding the syringe steady while the plunger is withdrawn with the right. The tourniquet is then at once removed, otherwise hæmatoma tends to form; thereafter the needle is withdrawn from the vein, and the contents of the syringe are at once emptied into the test-tube or flask of culture medium (the mouth of which must be flamed when the plug is removed), which is then plugged again immediately. The syringe and needle must be thoroughly washed out without delay. Another method is to insert a needle into the vein and to allow the blood to flow directly from it into the tube; this is satisfactory for obtaining blood for Wassermann reaction, etc., but is not satisfactory where sterility is essential. The wound may be sealed with gauze and collodion. In making blood cultures in order to detect the presence of enteric bacilli 5 c.c. of blood are added to 10 c.c. of sterilised ox bile or 50 c.c. of a 0·5 per cent. solution of bile salts in 1 per cent. sodium citrate. In examining for other organisms, *e g.* streptococci, 5 or 10 c.c. of blood are added to a large quantity of fluid medium (100 c.c. or more). Douglas and Colebrook recommend the addition to broth of trypsin, which both prevents clotting of the added blood and also destroys its anti-bacterial properties. They use a solution [1] of which 1 part is mixed with 20 parts of broth and tubed in amounts of 5 c.c., which are incubated at 37° C. for forty-eight hours, both aerobically and anaerobically, to control sterility. The mixture keeps in the ice-chest for several weeks; 1 c.c. of blood is added to 5 c.c. of trypsin broth. If it is desired to ascertain the number of living bacteria in the blood, the specimen should be mixed with an equal volume of a solution of ammonium oxalate 2 grams and sodium chloride 6 grams in 1000 c.c. of water, which is distributed in tubes and sterilised. Coagulation is thus prevented and known quantities of the blood can then be incorporated in melted agar and poured into Petri plates.

For obtaining the blood one uses a 10 c.c. syringe of "Record" or "all glass" type provided with a sharp needle, 5 cm. long and 1 to 1·22 mm. in external diameter; the separate parts should be placed in cold water and sterilised by boiling in plain water for five to ten minutes (they should not be brought into contact with any antiseptic or sodium carbonate). The parts of the syringe before use should be laid on a sterile surface (*e.g.* a sheet of paper which has been passed repeatedly through a Bunsen or spirit lamp flame) and should be similarly covered and then be put together by means of sterile forceps when cool (the needle and the nozzle of the syringe must not be touched with the fingers). The syringe should then be placed in a sterile container [2] and kept closed until required.

[1] Allen & Hanburys' "trypsin for bacteriological purposes."

[2] Metal cases with screw lids to accommodate "Record" syringes are supplied by the Surgical Manufacturing Co., 83–85 Mortimer Street, London, W.1.

When only a small sample of blood serum is required the following procedure may be employed. The circulation in the hand is stimulated, *e.g.* by immersion in hot water or by vigorous shaking of the arm. Then the skin of a finger at the base of the nail is swabbed with gauze moistened with spirit. A length of narrow tape is wound round the finger from the base to the terminal phalanx so as to congest the finger tip. Then a puncture through the cleansed skin is made with a sharp triangular needle which has been sterilised by flaming. The blood is collected in a piece of straight quill tube which has been drawn out to capillary ends and which is of such a length that it will fit into the bucket of the centrifuge. The tube is held almost horizontal during the process of filling. If blood ceases to flow a further quantity can usually be obtained without a second puncture by taking off and reapplying the tape When the tube is two-thirds full the empty part is warmed in a flame and the tip finally sealed. As this end cools the column of blood is retracted into the tube so that the other end can then be sealed. In this way several c c. of blood may be obtained. Separation of serum is aided by centrifuging the tube. Fig. 41 shows a Wright's capsule, in which one end is bent so that it can be attached to the limb of a centrifuge.

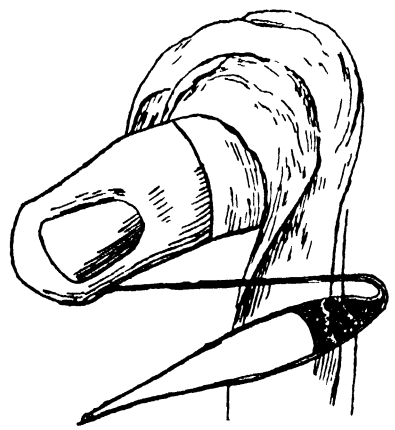


FIG. 41.—Wright's Blood-capsule, and method of filling same.

Cerebro-spinal Fluid — Lumbar Puncture.—This diagnostic procedure, which is often called for in cases of meningitis, can be carried out with a sterilised "antitoxin needle" as follows: The patient should lie on the right side, with knees somewhat drawn up and left shoulder tilted somewhat forward, so that the back is fully exposed. The skin over the lumbar region is then carefully cleansed and sterilised with alcohol and ether, and the hands of the operator should be thoroughly purified. The spines of the lumbar vertebræ having been counted, the left thumb or forefinger is pressed into the space between the third and fourth spines in the middle line; the needle is then inserted about half an inch to the right of the middle line at this level (or the needle may be inserted in the middle line) and pushed through the tissues, its course being directed slightly inwards and upwards, till it enters the subdural space. When this occurs, fluid passes along the needle, sometimes actually spurting out, and should be received in a sterile test-tube. Several cubic centimetres of fluid can thus usually be obtained, no suction being required; where there is no increase of pressure not more than 5–10 c.c. fluid should be removed. The depth of the subdural space from the surface varies from a little over an inch in children to 3 inches, or even more, in adults—the length of the needle must be suited accordingly. In making the puncture it is convenient to have either a sterile syringe attached, or to have the thick end of the needle covered with a pad of sterile wool, which is of course removed at once when the fluid begins to flow. It is advisable to use the platinum-iridium needles which are specially made for the

purpose, as a sudden movement of the patient may snap an ordinary steel needle; the needle should be of small bore, as larger needles inflict much pain.[1] The patient must be kept lying down for some hours afterwards.

Fæces.—Specimens intended for bacteriological examination should not be mixed with urine. For the detection of bacilli of the enterica and dysentery groups a loose motion must be obtained, and in the case of suspected carriers it may be necessary to administer a purgative, *e.g.* calomel. On the other hand, for the detection of cysts of Entamœba histolytica portions of mucus from the surface of solid motions are selected. Of a loose motion 1 c.c. is sufficient. The usual sterilised swab soaked in the fæces and replaced in its test-tube is satisfactory when there is no delay in making the examination. In other cases the fæces should be placed in a wide-mouthed 2-oz. bottle by means of a small metal spoon which is fitted into the cork for the purpose, the whole apparatus having been sterilised beforehand by autoclaving. In children especially, a convenient method is to pass a sterile glass speculum into the anus and through this introduce a swab rather stouter than the usual throat swab. When the making of cultures for organisms of the enterica and dysentery groups must be delayed for more than several hours after the specimen has been taken, it is advisable to add to one volume of the fæces two volumes of 30 per cent. neutral glycerin in 0·6 per cent. NaCl solution and to make a thorough mixture (Teague & Clurman). The presence of the glycerin prevents the suppression of the specific organisms by B. coli which would otherwise occur.

Secretions from Throat, etc.—Specimens of these secretions are obtained by rubbing the surface with a "swab" of cotton wool wrapped round the end of a strong wire. The swab is kept in a narrow test-tube of stout glass, and the other end of the wire is passed through the cork stopper The whole apparatus is sterilised by dry heat and kept ready for use. In taking specimens from the throat it is important that no antiseptic should have been applied (*e.g.* as a gargle) for some hours before.

Secretion from the Naso-pharynx.—A specimen of pharyngeal mucus may be obtained by means of a swab of cotton wool on the end of a metal wire. The wire ought to be longer than that used in the case of a throat swab and bent near the extremity. A tongue depressor is used; the wire is introduced into the mouth, and passed up behind the soft palate and then brought into contact with the posterior pharyngeal wall. Care must be taken not to touch any part of the mucous membrane of the mouth. The best method, however, is by means of West's tube. This consists of a glass tube shaped like a catheter, in the interior of which is a thin wire bearing the swab, the latter being just within the end of the tube The bend of the tube can be used to depress the tongue, or a depressor may be used, and when the tube is sufficiently introduced it is turned up behind the soft palate, and the end of the wire is pressed so as to protrude the swab, which is then brought into contact with the pharyngeal wall. The swab is then drawn back into the tube (in fact, it usually springs back) and the tube is removed from the mouth. It is important to avoid contamination with mouth

[1] External diameter about 0·04 inch, *i.e.* B.W.G. 57–60.

organisms, which tend to inhibit the growth of meningococci. Cultures for the latter must be made at once and placed in the incubator as quickly as possible, as cold has an injurious effect on the organisms. If the inoculations have been made at some distance from the laboratory, the plates should be carried in an apparatus with a hot-water jacket—a bag containing a hot-water bottle is often sufficient.

Urine.—In such an examination care must be taken to prevent the contamination of the urine by extraneous organisms. In the male, specimens withdrawn by a sterile catheter into a sterile vessel are preferable, but it is often sufficient to wash thoroughly the glans penis and the meatus with 1 : 1000 corrosive sublimate—the lips of the meatus being everted for more thorough cleansing; the urine is then passed into a series of sterile flasks, the first of which is rejected in case contamination has occurred. In the female, after similar precautions as regards external cleansing, a sterile catheter must be used. If organisms are scanty, the specimen should be centrifuged and films and cultures made from the sediment. Sometimes in examining for tubercle bacilli it may be advisable to collect the twenty-four hours' urine in the ordinary way and to remove some of the sediment which has settled and concentrate the latter in the centrifuge.

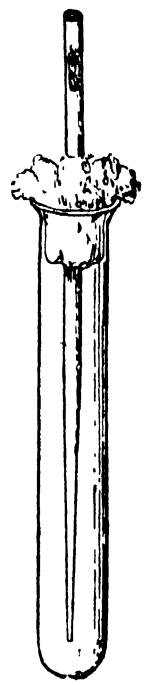


FIG. 42.—Test-tube and pipette arranged for obtaining fluids containing bacteria.

Post-mortem Material.—Fluids from the body cavities, pus, urine, etc., may be secured with sterile pipettes (see Fig. 42). The end of the pipette should be plugged with a small piece of cotton wool which is inserted before sterilising; it prevents the accidental sucking up of infective material.

Solid organs to be examined should, if possible, be obtained whole. They may be treated in one of two ways. (1) The surface over one part about an inch broad is seared with a cautery heated to dull red heat. All superficial organisms are thus killed. An incision is made in this seared zone with a sterile scalpel, and small quantities of the juice are removed by a platinum spud to make cover-glass preparations and plate or smear cultures (a piece of quill tube drawn out in the flame till slightly narrowed at one end which is then broken across so as to leave a jagged end is an excellent substitute for the spud). (2) An alternative method is as follows: The surface is sterilised by soaking it well with 1 : 1000 corrosive sublimate for half an hour. It is then dried, and the capsule of the organ is cut through with a sterile knife, the incision being further deepened by tearing. In this way a perfectly uncontaminated surface is obtained. The clinical history of the case will often suggest what the procedure ought to be in examination.

CHAPTER V

RELATIONS OF BACTERIA TO DISEASE—THE PRODUCTION OF TOXINS BY BACTERIA

Introductory.—It has already been stated that a strict division of micro-organisms into true *parasites* and *saprophytes* cannot be made. No doubt there are organisms, such as the bacillus of tubercle, gonococcus, etc., which are in natural conditions always parasites associated with disease. But these can lead a saprophytic existence in specially prepared conditions, and there are many of the disease-producing organisms, such as the organisms of typhoid and cholera, which can flourish readily outside the body, even in ordinary conditions. A similar statement applies to the terms *pathogenic* and *non-pathogenic*. By the term pathogenic is meant the power which an organism has of producing morbid changes or effects in the animal body, either under natural conditions or in conditions artificially arranged, as in direct experiment. Now we know of no organisms which will in all circumstances produce disease in all animals, and, on the other hand, many bacteria described as harmless saprophytes will produce pathological changes if introduced in sufficient quantity. When, therefore, we speak of a pathogenic organism, the term is merely a relative one, and indicates that in certain circumstances the organism will produce disease, though in the science of human pathology it is often used for convenience as implying that the organism produces disease in man in *natural* conditions. The term '*commensal*' is applied to organisms normally present as saprophytes on the skin or mucous surfaces of the body. Certain commensals, *e.g.* staphylococci and streptococci, have the capacity under certain conditions of invading the tissues and producing pathogenic effects. This is a matter of importance in connection with the origin of natural infections.

Modifying Conditions.—In studying the pathogenic effects in any instance, both the micro-organisms and the animal affected must be considered, and not only the species of each, but also

its exact condition at the time of infection. In other words, the resulting disease is the product of the sum-total of the characters of the infecting agent, on the one hand, and of the subject of infection, on the other. We may, therefore, state some of the chief circumstances which modify each of these two factors involved, and, consequently, the diseased condition produced.

1. *The Infecting Agent.*—In the case of a particular species of bacterium its effect will depend chiefly upon (*a*) its virulence and (*b*) the number introduced into the body. To these may be added (*c*) the path of infection.

The *virulence, i.e.* the power of multiplying in the body and producing disease, varies greatly in different conditions, and the methods by which it can be diminished or increased will be afterwards described (*vide* Chapter VI.). One important point is that when a bacterium has been enabled to invade and multiply in the tissues of an animal, its virulence for that species is often increased. This is well seen in the case of certain bacteria which are normally present on the skin or mucous surfaces. Thus it has been repeatedly proved that the B. coli cultivated from a septic peritonitis is much more virulent than that taken from the bowel of the same animal. The virulence may be still more increased by inoculating from one animal to another in series—the method of *passage.* Widely different effects are, of course, produced on the virulence being altered. For example, a streptococcus which produces merely a local inflammation or suppuration, may produce a rapidly fatal septicæmia when its virulence is raised. Virulence also has a relation to the animal employed, as occasionally on being increased for one species of animal it is diminished for another. For example, streptococci, on being inoculated in series through a number of mice, acquire increased virulence for these animals, but become less virulent for rabbits (Knorr). Certain facts suggest that there may be a periodicity in virulence, *i.e.* that an organism may for a time produce a relatively mild type of disease and then develop into a more potent strain capable of overcoming the resistance of a greater number of individuals ; this would account for the fact that in the case of some diseases widespread epidemics occur at almost fixed intervals of years. The theoretical consideration of virulence must be reserved for a later chapter (see Immunity).

The *number* of the organisms introduced, *i.e.* the dose of the infecting agent, is another point of importance. The healthy tissues can usually resist a certain number of pathogenic

organisms of given virulence, a good example being often furnished in ulcerative endocarditis in man, where bacteria may be shed into the blood for a considerable time without causing secondary lesions. It is only in a few instances that one or two organisms introduced will produce a fatal disease, *e.g.* the case of anthrax in white mice. The healthy peritoneum of a rabbit can resist and destroy a considerable number of pyogenic micrococci without any serious result, but if a larger dose be introduced, a fatal peritonitis may follow. There is, therefore, for a particular animal, a minimum lethal dose which can be determined by experiment only—a dose, moreover, which is modified by various circumstances difficult to control.

The path of infection may alter the result, serious effects often following entrance into the blood stream. Staphylococci injected subcutaneously in a rabbit may produce only a local abscess, whilst on intravenous injection multiple abscesses in certain organs may result and death may follow. Local inflammatory reaction with subsequent destruction of the organisms may be restricted to the site of infection or may occur also in the related lymphatic glands. The latter therefore act as a second barrier of defence, or as a filtering mechanism which aids in protecting against blood infection. This is well illustrated in the case of "poisoned wounds." In some other cases, however, the organisms are very rapidly destroyed in the blood stream, and Klemperer found that, in the dog, subcutaneous injection of the pneumococcus produces death more readily than intravenous injection. In the case of syphilis, inoculation of monkeys is more successful by scarification than by any other means. When the organisms are introduced into the subcutaneous tissue, for example, they are killed off. In the case of the anthrax bacillus, infection occurs more readily by scarification than by subcutaneous injection.

2. *The Subject of Infection.*—Amongst healthy individuals susceptibility to a particular microbe may vary according to (*a*) species, (*b*) race and individual peculiarities, (*c*) age. Different species of the lower animals show the widest variation in this respect, some being extremely susceptible, others highly resistant. Then there are diseases, such as syphilis, etc., which under natural conditions are peculiar to the human subject and can only be transmitted to a few of the lower animals. And again, there are others, the typical lesions of which cannot be experimentally reproduced in animals, or appear only imperfectly, although pathogenic effects follow inoculation with the

organisms. In the case of the human subject, differences in susceptibility to a certain disease are found amongst different races, and also amongst individuals of the same race, as is well seen in the case of tubercle and other diseases. Age also plays an important part, young subjects being more liable to certain diseases, *e.g.* to diphtheria. Further, at different periods of life certain parts of the body are more susceptible; for example, in early life, the bones and joints to tubercular and acute suppurative affections.

In increasing the susceptibility of a given individual, conditions of *local or general diminished vitality* play the most important part. It has been experimentally proved that conditions such as exposure to cold, fatigue, starvation, etc., all diminish the natural resistance to bacterial infection. Rats naturally immune to glanders can be rendered susceptible by being fed with phloridzin, which produces a sort of diabetes. Also a local susceptibility may be produced by injuring or diminishing the vitality of a part. If, for example, previous to an intravenous injection of staphylococci, the aortic cusps of a rabbit be injured, the organisms may settle there and set up an ulcerative endocarditis; or if a bone be injured, they may produce suppuration at the part, whereas in ordinary circumstances these lesions would not take place. In addition to such conditions as have been mentioned, there are also some special factors which predispose to infection. In the case of silicosis of the lungs a marked liability to infection with the tubercle bacillus has long been recognised, and recently it has been shown by the experimental work of Kettle and Gye that silica has a special effect in making the tissues susceptible to invasion by this organism. Another striking example is the remarkable effect which ionisable calcium salts have in leading to infection with the B. welchii and other anaerobes, as was first observed by Gye and Cramer. They showed that when, for instance, the B. welchii free of its toxin is injected into a mouse or guinea-pig it rapidly becomes destroyed by lysis or by phagocytosis, whereas if some solution of calcium chloride is injected along with the organism or its spores, a spreading gas gangrene results. A similar effect may follow, though not so readily, when the calcium salt is injected at some part of the body other than the site of injection of the bacilli. In contrast to this, salts of magnesium, sodium, potassium, etc., are devoid of such favouring effects. Calcium salts accordingly seem to have a special action in breaking down the defences of the tissues or leading to a state of "kataphylaxis" as these workers have called it. These results suggest that other

specific factors as yet unknown may be concerned in the origin of other infections occurring naturally.

Such facts, established by experiment (and many others might be given), illustrate the important part which local or general conditions of diminished vitality may play in the production of disease in the human subject. This has long been known by clinical observation. In normal conditions the blood and tissues of the body, with the exception of the skin and certain of the mucous surfaces, are bacterium-free, and if a few organisms gain entrance, they are destroyed. But if the vitality becomes lowered, their entrance becomes easier and the possibility of their multiplying and producing disease greatly increased. In this way the favouring part played by fatigue, cold, etc., in the production of diseases of which the direct cause is a bacterium may be understood. It is important to keep in view in this connection that many of the inflammation-producing and pyogenic organisms are normally present on the skin and various mucous surfaces; and also that during epidemics of a disease, *e.g.* typhoid, cholera, meningitis, diphtheria, the pathogenic organisms may be present on the mucous membranes of healthy individuals—that is, may have gained access to the body without producing the disease. The action of a certain organism may devitalise the tissues to such an extent as to pave the way for the entrance of other bacteria; we may mention the liability to the occurrence of pneumonia, erysipelas, and various suppurative conditions, in the course of or following infective fevers. In some cases the specific organism may produce lesions through which the other organisms gain entrance, *e.g.* in typhoid, diphtheria, etc. A notable example of diminished resistance to bacterial infection is seen in the case of diabetes; tuberculosis and infection with pyogenic organisms are prone to occur in this disease, and are apt to be of a severe character. It is not uncommon to find in the bodies of those who have died from chronic wasting disease, collections of micrococci or bacilli in the capillaries of various organs, which have entered in the later hours of life—that is to say, the bacterium-free condition of the blood has been lost in the period of prostration preceding death.

The methods by which the natural resistance may be specifically increased belong to the subject of immunity, and are described in the chapter on that subject.

Carriers.—As has been stated, many of the organisms which produce inflammatory and suppurative affections are normally present on the skin or mucous membranes; but it has also been

established that several of the causal agents of acute infectious diseases, such as typhoid, epidemic meningitis, diphtheria, etc., may flourish on the mucous membranes of individuals in apparent health. Such individuals are known as "carriers," and they play a highly important part in the spread of these infections. It will be shown more fully below that many of the pathogenic bacteria, as regards their morphological and cultural characters, are very similar to, and have apparently sprung from, organisms which are normally present in the sites of the several lesions. Thus the whole typhoid-dysentery group are related to the B. coli, and the meningococci to various Gram-negative diplococci which abound in the naso-pharynx. The carrier state may thus be regarded, in a sense, as a return of these organisms to a saprophytic existence. One group of carriers is constituted by those who have suffered from the disease, and in whom the organism persists after recovery, and these are usually designated "temporary" or "chronic" according to the duration of the condition. The proportion of those who become carriers, and the period of carrying the organisms, vary in different diseases. In cholera, for example, the period is usually comparatively short, whereas in typhoid the organisms not infrequently persist for an indefinite period of time. The other group of carriers comprises apparently healthy individuals, who harbour the organisms and are not known to have suffered from the disease. Some of these may really have had a mild attack, but in others there is no evidence of this; a few subsequently develop the disease—"precocious" carriers. In many instances previous contact with a case of the disease can be traced; this is a usual occurrence with typhoid carriers. On the other hand, no connection of this kind may be discoverable; for instance, the meningococcus is often found, especialy during epidemics, in "non-contacts," the organism apparently spreading widely from individual to individual in the community. Further facts will be given in connection with the special diseases.

Modes of Bacterial Action.—In the production of disease by micro-organisms there are two main factors involved, namely, (*a*) the multiplication of the living organisms after they have entered the body, and (*b*) the production by them of poisons which may act both upon the tissues around and upon the body generally. The former corresponds to *infection*, the latter is of the nature of *intoxication* or poisoning. In different diseases one of these is usually the more prominent feature, but both are always more or less concerned.

1. *Infection and Distribution of the Bacteria in the Body.*—After pathogenic bacteria have invaded the tissues, or in other words, after infection by bacteria has taken place, their further behaviour varies greatly in different cases. In the human subject after infection one of two things usually happens. In the first place, the organisms may remain local, producing little reaction around them, as in tetanus, or a well-marked lesion, as in diphtheria, etc. Or in the second place, they may pass by the lymph or blood stream to other parts or organs in which they settle, multiply, and produce lesions, as in tubercle. In certain cases they may reach and multiply in the blood stream, producing a fatal septicæmia. In the lower animals this multiplication of the organisms in the blood throughout the body may be very extensive (for example, the septicæmia produced by the pneumococcus in rabbits); but in septicæmia in man it is seen in less degree, the organisms rarely remain in large numbers in the circulating blood, and their detection in it during life by microscopic examination is rare, and even culture methods may give negative results unless a large amount of blood is used. In such cases, however, the organisms may be found *post mortem* lying in large numbers within the capillaries of various organs, *e.g.* in cases of septicæmia produced by streptococci. It is important to draw a distinction between the mere presence of organisms in the blood—*bacteriæmia*, and their active multiplication in it—*septicæmia*. The former condition represents merely an overflow of the organisms from the lesions, as is well exemplified in the early stages of typhoid fever. It is also a common occurrence in subacute ulcerative endocarditis where organisms may be cultivated from the blood over a considerable period. But there is no progressive multiplication in such cases, and we may say that in bacteriæmia the organisms would soon disappear if the source of supply were removed.

2. *Production of Chemical Poisons.*—In all these cases the growth of the organisms is accompanied by the formation of *chemical products*, which act generally or locally in varying degree as toxic substances. The toxic substances become diffused throughout the system, and their effects are manifested chiefly by symptoms such as the occurrence of fever, disturbances of the circulatory, respiratory, and nervous systems, etc. In some cases corresponding changes in the tissues are found; for example, the changes in the nervous system in diphtheria, to be afterwards described. The general toxic effects may be so slight as to be of no importance, as in the case of a local suppura-

tion; or they may be very intense, as in pyæmia; or, again, less severe but producing cachexia by their long continuance, as in tuberculosis.

The occurrence of *local tissue changes or lesions* produced in the neighbourhood of the bacteria, as already mentioned, is one of the most striking results of bacterial action, but these also must be traced to chemical substances formed in or around the bacteria, and either directly or through the medium of ferments. In this case it is more difficult to demonstrate the mode of action, for in the tissues the chemical products are formed by the bacteria slowly, continuously, and in a certain degree of concentration, and these conditions cannot be exactly reproduced by experiment. Further, it is very doubtful whether all the chemical substances formed by a certain bacillus growing in the tissues are also formed by it in cultures outside the body (*vide* p. 165). The separated toxin of diphtheria, like various vegetable and animal toxins, however, possesses a local toxic action of very intense character, evidenced often by extensive necrotic change. It is also to be noted that more than one poison may be produced by a given bacterium, *e.g.* the tetanus bacillus (p. 533).

The injection of large quantities of many different pathogenic organisms in the *dead* condition results in the production of a local inflammatory change which may be followed by suppuration, this effect being possibly brought about by certain substances in the bacterial protoplasm common to various species, or at least possessing a common physiological action (Buchner and others). When dead tubercle bacilli, however, are introduced into the blood stream, nodules do result in certain parts which have a resemblance to ordinary tubercles. In this case the bodies of the bacilli evidently contain a highly resistant and slowly acting substance which gradually diffuses around and produces effects (*vide* Tuberculosis).

Summary.—We may say, then, that the action of bacteria as disease-producers, as in fact their power to exist and multiply in the living body, depends upon the chemical products formed directly or indirectly by them. This action is shown by *tissue changes* produced in the vicinity of the bacteria or throughout the system, and by *toxic symptoms* of great variety of degree and character.

We shall first consider the effects of bacteria on the body generally, and afterwards the nature of the chemical products.

Effects of Bacterial Action

These may be for convenience arranged in a tabular form as follows:

A. Tissue Changes.

(1) Local changes, *i.e.* changes produced in the neighbourhood of the bacteria.

Position: (*a*) At primary lesion.
(*b*) At secondary foci.

Character: (*a*) Tissue reactions } Acute or
(*b*) Degeneration and necrosis } chronic.

(2) Changes produced at a distance from the bacteria, directly or indirectly, by the absorption of toxins.

(*a*) In special tissues—
(α) as the result of damage, *e.g.* nerve cells and fibres, secreting cells, vessel walls, or
(β) changes of a reactive nature in the blood-forming tissues and organs.
(*b*) General anatomical changes, the effects of malnutrition or of increased waste.

B. Symptoms and Changes in Metabolism.
The occurrence of fever, of errors of assimilation and elimination, etc.

A. Tissue Changes produced by Bacteria.—The effects of bacterial action are so various as to include almost all known pathological changes. However varied in character, they may be classified under two main headings: (*a*) those of a degenerative or necrotic nature, the direct result of damage; and (*b*) those of reactive nature, defensive or reparative. The former are the expression of the necessary vulnerability of the tissues, the latter of protective powers evolved for the benefit of the organism. In the means of defence both leucocytes and the fixed cells of the tissues are concerned. Both show phagocytic properties, *i.e.* have the power of taking up bacteria into their protoplasm. The cells are guided towards the focus of infection by chemotaxis, and thus we find that different bacteria may attract different cells. The most rapid and abundant supply of phagocytes is seen in the case of suppurative conditions where the

polymorphonuclear (neutrophile) leucocytes of the blood are chiefly concerned. When the local lesion is of some extent there is usually an increase of these cells in the blood—a neutrophile leucocytosis. And further, observation has shown that associated with this there is in the bone-marrow an increased number of the mother-cells of these leucocytes—the neutrophile myelocytes. The passage of the neutrophile leucocytes from the marrow into the blood, with the resulting leucocytosis, is also apparently due to the absorbed bacterial toxins acting chemotactically on the marrow. These facts abundantly show that the means of defence is not a mere local mechanism, but that increased proliferative activity in distant tissues is called into play. In addition to direct phagocytosis by these leucocytes, there is also evidence that an important function is the production in the body of bactericidal and other antagonistic substances. In other cases the cells chiefly involved are the mononuclear hyaline leucocytes, and with them the endothelial cells, adventitial cells, etc.; these form together a group of non-granular cells to which Metchnikoff gave the name *macrophages*. Reaction by such cells is well seen in typhoid fever, where the specific bacillus appears to have little or no action on the neutrophile leucocytes. In other cases, again, the reaction may be mainly on the part of the connective tissue cells, though their proliferation is always associated with some variety of leucocytic infiltration and usually also with the formation of new blood vessels. Such a connective tissue reaction occurs especially in slow infections or in the later stages of an acute infection. The reactive tissue changes in the presence of bacterial invasion are naturally very varied,—examples of this will be found in subsequent chapters,—but they may be said to be manifestations of the two fundamental processes of (*a*) increased functional activity—movement, phagocytosis, secretion, etc.—and (*b*) increased formative activity—cell growth and division. The exudation from the blood vessels has been variously interpreted. There is no doubt that the exudate may have bactericidal or opsonic properties and also acts as a diluting agent, but it must still be held as uncertain whether the process of exudation ought to be regarded as primarily defensive or as the direct result of damage to the endothelium of the vessels. It may also be pointed out that the various changes referred to are none of them peculiar to bacterial invasion; they are examples of the general laws of tissue change under abnormal conditions, and they can all be reproduced by chemical substances in solution or in a particulate state. What constitutes

their special feature is their progressive or spreading nature, due to the bacterial multiplication.

(1) Local Lesions.—In some diseases the lesion has a special site; for example, the lesion of typhoid fever, and, to a less extent, that of diphtheria. In other cases it depends entirely upon the point of entrance, *e.g.* malignant pustule and the conditions known as wound infections. In others again, there is a special tendency for certain parts to be affected, as the upper parts of the lungs in tubercle. In some cases the site has a mechanical explanation.

When organisms gain an entrance to the blood from a primary lesion, the organs specially liable to be affected vary greatly in different diseases. Pyogenic cocci show a special tendency to settle in the capillaries of the kidneys and produce miliary abscesses, whilst these lesions rarely occur in the spleen. On the other hand, the nodules in disseminated tubercle or glanders are much more numerous in the spleen than in the kidneys, which in the latter disease are usually free from them. The distribution of the lesions thus cannot be explained on a mechanical basis.

Acute Local Lesions.—The local inflammatory reaction presents different characters in different conditions. It may be accompanied by abundant fibrinous exudation, or by great catarrh (in the case of an epithelial surface), or by hæmorrhage, or by œdema; it may be localised or spreading in character; it may be followed by suppuration, and may lead up to necrosis of the tissues of the part, a good example of the latter event being a furuncle. Examples will be given in subsequent chapters. The necrotic or degenerative changes affecting especially the more highly developed elements of tissues are chiefly produced by the direct action of the bacterial poisons, though aided by the disturbances of nutrition involved in the vascular phenomena. It may here be pointed out that a well-marked inflammatory reaction is often found in animals which occupy a medium position in the scale of susceptibility, and that an organism which causes a general infection in a certain animal may produce only a local inflammation when its virulence is lessened.

Chronic Local Lesions.—In a considerable number of diseases produced by bacteria the local tissue reaction is a more chronic process than that described; there is less vascular disturbance and a greater preponderance of the proliferative processes, leading to new formation of connective tissue. This formation may occur in foci here and there, so that nodules result, or it may be

more diffuse. Such changes especially occur in the diseases often known as the *infective granulomata*, of which tubercle, syphilis, leprosy, glanders, actinomycosis, etc., are examples.

(2) General Lesions produced by Toxins.—In the various infective conditions produced by bacteria, changes commonly occur in certain organs unassociated with the presence of the bacteria; these are produced by the action of bacterial products circulating in the blood. Many such lesions can be produced experimentally. The secreting cells of various organs, especially the kidney and liver, are specially liable to change of this kind. Cloudy swelling, which may be followed by fatty change or by actual necrosis with granular disintegration, is common. Hyaline change in the walls of arterioles may occur, and in certain chronic conditions amyloid change is brought about in a similar manner. The latter has been produced in animals by repeated injections of the Staphylococcus aureus. Capillary hæmorrhages are not uncommon, and are in many cases due to an increased permeability of the vessel walls, aided by changes in the blood plasma, as evidenced sometimes by diminished coagulability. Similar hæmorrhages may follow the injection of some bacterial toxins, *e.g.* of diphtheria, and also of vegetable poisons, *e.g.* ricin and abrin. Skin eruptions occurring in the exanthemata are probably produced in the same way, though in many of these diseases the causal organism has not yet been isolated. We have, however, the important fact that corresponding skin eruptions may be produced by poisoning with certain drugs. In the nervous system degenerative changes have been found in diphtheria, both in the spinal cord and in the peripheral nerves, and have been reproduced experimentally by the products of the diphtheria bacilli. It is probable that some of the lesions of the nervous system occurring in syphilis have likewise a toxic origin. Besides the effects on tissues enumerated, more subtle changes, *e.g.* those observed in the reaction and coagulability of the blood, are also probably traceable to toxic effects.

B. Disturbances of Metabolism, etc.—It will easily be realised that such profound tissue changes as have been detailed cannot occur without great interference with the normal bodily metabolism. General malnutrition and cachexia are of common occurrence, and it is a striking fact found by experiment that after injection of bacterial products, *e.g.* of the diphtheria bacillus, a marked loss of body weight often occurs which may be progressive, leading to the death of the animal. In bacterial disease assimilation is often imperfect, for the digestive glands

are affected, it may be by actual poisoning by bacterial products, it may be by the occurrence of fever, and excretion is interfered with by the damage done to the excretory cells. But of all the changes in metabolism the most difficult to understand is the occurrence of that interference with the heat-regulating mechanism which results in fever. The degree and course of the latter vary, sometimes conforming to a more or less definite type, when the bacilli are selective in their field of operation, as in croupous pneumonia or typhoid, sometimes being of a very irregular kind, especially when the bacteria from time to time invade fresh areas of the body, as in pyæmic affections. The main point of interest regarding the development of fever is as to whether it is a direct effect of the circulation of bacterial toxins, or if it is to be looked on as part of the reaction of the body against the irritant. This question has still to be settled, and all that we can do is to adduce certain facts bearing on it. Thus in diphtheria and tetanus, where toxic action leading to degeneration plays such an important part, fever may be a very subsidiary feature, except in the terminal stage of the latter disease ; and in fact in diphtheria profoundly toxic effects may be produced with little or no interference with heat regulation. On the other hand, in bacterial diseases where defensive and reparative processes predominate, fever is rarely absent, and it is nearly always present when there is an active leucocytosis going on. In this connection it may be remarked that several observers have found that, when a relatively small amount of the dead bodies of certain bacteria are injected into an animal, fever occurs ; while the injection of a large amount of the same is followed by subnormal temperatures and rapidly fatal collapse. It might appear as if this indicated that the occurrence of fever had a beneficial effect, but this is one of the points at issue. Certainly such an effect is not due to the bacteria being unable to multiply at the higher degrees of temperature occurring in fever, for this has been shown not to be the case. A certain amount of evidence has been brought forward to show that antibodies are more rapidly produced when the temperature of the body is above the normal, and it has been supposed that in this way fever may be of the nature of a defensive reaction. It must be borne in mind, however, that in fever there is not merely elevation of temperature but also a disturbance of metabolism with increased heat production and heat loss ; mere elevation of temperature might be attained without this metabolic disturbance. At present it is not possible to say whether fever should be regarded as essentially a beneficial process. A pro-

duction of antagonistic substances may certainly be effected without the occurrence of fever or of any apparent disturbance of health.

Symptoms.—Many of the symptoms occurring in bacterial infections are produced by the histological changes mentioned, as can be readily understood; whilst in the case of others, corresponding changes have not yet been discovered. Of the latter, those associated with fever, with its disturbances of metabolism and manifold affections of the various systems, are the most important. The nervous system is especially liable to be affected—convulsions, spasms, coma, paralysis, etc., being common. The symptoms due to disturbance or abolition of the functions of secretory glands also constitute an important group, forming, as they do, a striking analogy to what is found in the action of various drugs.

These tissue changes and symptoms are given only as illustrative examples, and the list might easily be greatly amplified. The important fact, however, is that *nearly all, if not quite all, the changes found throughout the organs (without the actual presence of bacteria), and also the symptoms occurring in infective diseases, either can be experimentally reproduced by the injection of bacterial poisons or have an analogy in the action of drugs.*

The Toxins produced by Bacteria

Early Work on Toxins.—The first to study systematically the production of bacterial toxins was Brieger, who isolated from putrefying substances, and also from bacterial cultures, nitrogen-containing bodies, which he called *ptomaines*. Similar bodies occurring in the ordinary metabolic processes of the body had previously been described and called *leucomaines*. Ptomaines isolated from pathogenic bacteria in no case reproduced the symptoms of the disease. The methods by which they were isolated were faulty, and they have therefore only historic interest. The introduction of the principle of rendering fluid cultures bacteria-free by filtration through unglazed porcelain, and its application by Roux and Yersin to obtain, in the case of the B. diphtheriæ, a solution containing a toxin which reproduced the symptoms of this disease (*vide* pp. 462, 472), constitute the starting-point of modern work on the subject.

General Facts regarding Bacterial Toxins.—In dealing with the action of toxins it is necessary to distinguish between the effects produced by the actual constituents of the bacterial

protoplasm (intrabacterial toxins, endotoxins) and those which in a few bacteria are traceable to soluble substances passing out into the media in which these bacteria may be growing (extrabacterial toxins, exotoxins). The former are concerned in the action of by far the greater number of pathogenic bacteria ; the latter account for the pathogenic processes seen in a limited number of diseases, of which diphtheria and tetanus are the most important. This distinction is important as, in consequence of these last two diseases having specially been investigated early in the history of research on the subject, there has been a tendency to take for granted that poisons of a similar constitution are concerned in all cases of bacterial intoxication. Such an assumption is not justified by facts, and we do not even know whether the endotoxins and exotoxins belong to the same group of chemical bodies. The terms are merely used as a convenient means of indicating certain differences between the two groups of toxic bodies.

Endotoxins.—The dead bodies of certain bacteria have been found to have toxic properties. When, for instance, tubercle bacilli are killed by heat and injected into the body tissues of a susceptible animal, tubercular nodules are found to develop round the sites where they have lodged. From this it is inferred that they must have contained characteristic toxins, seeing that characteristic lesions result. The bodies of such organisms as the pyogenic cocci, the B. typhosus, B. dysenteriæ (Shiga), and the V. choleræ, likewise give rise to pathogenic effects. Such toxins appear to occur in the fluids in which the bacteria are living, chiefly as a result of the disintegration of the organisms, which is always occurring in any bacterial growth. The death of bacteria occurs also in the body of an infected animal, and the dissolution of the dead bacteria constitutes an important means by which the poisons they contain are set free. There is evidence that during growth bacteria often form poisons which are hurtful to their own vitality, and also that ferments are produced by them which have a solvent effect on the poisoned members of the colony. Such a process of *autolysis*, as it has been called, may have an important result in liberating endotoxins. Our knowledge concerning the action of such toxins is chiefly derived from the study of the effects produced by injecting into animals either the bodies of bacteria (killed by chloroform vapour or by heat) or bacterial protoplasm disintegrated mechanically or artificially autolysed. The effects produced by such injections do not present in any particular case specific characters, though this is in conformity with the fact that in

many different infections the symptoms have a certain community in character.

In connection with endotoxins the work of Vaughan is of importance. After treating bacteria with alcohol and ether he finds that on further treatment with alkaline alcohol (2 per cent. sodium hydrate in alcohol) certain groups are split off from the bacterial protoplasm leaving a substance which he calls "poisonous protein." This substance gives the biuret reaction, reacts as a protein with Millon's reagent, but is soluble in alcohol; he considers that it is probably a polypeptid. He obtained poisons of closely similar nature from a variety of bacteria, as well as from proteins from other sources, and considers that in infections the poison is set free by disintegration of bacteria. The effects produced by the poisonous proteins are of similar nature to those produced by the dead bacteria, but occur almost at once. Whilst these results are of interest in showing the presence of toxic molecules of relatively small size in the bacterial protoplasm, it does not appear justifiable to conclude that such molecules are the same as those set free in the solution of bacteria within the tissues.

Aggressins.—In certain cases there is difficulty in understanding the action of bacteria which neither form soluble toxins in a fluid medium nor possess a highly toxic protoplasm, and which yet produce effects at a distance from the focus of infection, *e.g.* B. anthracis. To explain such occurrences it has long been regarded as a possibility that such bacteria produce toxins only within the animal tissues, and it has further been thought possible that bacteria, such as, for example, the typhoid bacillus, which do distribute into media intracellular toxins, might either produce these toxins more readily in the tissues or might produce in addition other toxins of a different nature. During recent years such toxins have been much studied, and the name *aggressins* has been given to them. The evidence adduced for the existence of these aggressins as a separate group of bacterial poisons is of the following kind: An animal is killed by a dose of the typhoid, dysentery, cholera, or tubercle bacillus, or by a staphylococcus, the organism being introduced into one of the serous cavities. After death the serous exudate, which in all these cases is present, is taken, and centrifuged to remove the bacteria so far as this can be done by such a procedure; the bacteria which are left are killed by shaking the fluid up with toluol and leaving it to stand for some days. Such a fluid, in a dose which by itself has no pathogenic effect, has the property of transforming a non-lethal dose of the bacterium used into

one having fatal effect. Further, the effects of the combined actions of the bacteria and aggressins are often of a much more acute character than can be obtained with toxic products developed *in vitro*. The effects produced by aggressins are attributed to a paralysing action on the phagocytic functions of the leucocytes. The subject is full of difficulties, and in the case of certain of the organisms employed results similar to those attributed to aggressin action have been observed with the fluid obtained by macerating living cultures—the deduction being that in the aggressins we are merely dealing with a particular type of endotoxin. As evidence of the existence of a special group of toxins, it has been stated that a special type of immunity against the aggressins can be originated. Perhaps the most important aspect of the controversy is the recognition of the existence of toxins having an action on the leucocytes. A poison causing death of these cells in connection with the pus-forming action of the pyogenic cocci has been described under the name of leucocidin, and Eisenberg records that in *in vitro* mixtures of leucocytes and cultures of the bacillus of symptomatic anthrax loss of motility and degeneration of the cells may be observed.

The non-specific effects of endotoxins are responsible for the general changes occurring in the greater number of the common bacterial infections in man.

Exotoxins.—In the cases of a few pathogenic bacteria the media in which they are growing become extremely toxic. This is more marked in some cases than in others. The best examples of bacteria thus producing soluble toxins are the diphtheria, tetanus, and botulismus bacilli ; in these and similar cases when bouillon cultures are filtered bacterium-free by means of a porcelain filter, toxic fluids are obtained, which on injection into animals may reproduce the characteristic symptoms of the corresponding diseases. This contrasts with such cases as those of the pneumococcus or of B. anthracis, filtered cultures of which are usually non-toxic. Poisons appearing in culture media have been called extracellular toxins or *exotoxins*, but we cannot as yet say whether they are excreted by the bacteria or whether they are produced by the bacteria acting on the constituents of the media. The exotoxins are easily obtainable in large quantity and high concentration, but no method has been discovered of obtaining them in a pure form, and our knowledge of their properties is exclusively derived from the study of the toxic filtrates of bouillon cultures—these filtrates being usually referred to simply as the "toxins." These toxins differ in

their effects from the endotoxins in that specific actions on certain tissues are often manifested. Thus the toxins of the diphtheria, the tetanus, and the botulismus bacilli all act on the nervous system ; with some of the pyogenic bacteria, on the other hand, poisons, probably of similar nature, produce solution of red blood corpuscles, which may in certain instances explain the anæmia so common in the associated diseases. In the action of many of these toxins the occurrence of a period of incubation between the introduction of the poison into the animal tissues and the appearance of symptoms is often a marked feature.

Amongst the properties of the exotoxins are the following. They are apparently all uncrystallisable ; they are soluble in water and they are little dialysable, though they vary in this respect ; they are precipitated along with proteins by concentrated alcohol, and also by ammonium sulphate ; if they are proteins they are either albumoses or allied to the albumoses ; they are often relatively unstable, having their toxicity diminished or destroyed by heat (the degree of heat which is destructive varies much in different cases), light, and by certain chemical agents. Their potency is often altered in the precipitations practised to obtain them in a pure or concentrated condition, but among the precipitants ammonium sulphate has little if any harmful effect. Regarding the endotoxins we know much less, but it is probable that, chemically, their nature is similar, though most of them are not so easily injured by heat, *e.g.* those of the tubercle bacillus, already mentioned. In the case of all toxins the fatal dose for an animal varies with the species, body-weight, age, and previous conditions as to food, temperature, etc. In estimating the minimal lethal dose of a toxin these factors must be carefully considered.

The following is the best method of obtaining concentrated exotoxins : The toxic fluid is placed in a shallow dish, and ammonium sulphate crystals are well stirred in till no more dissolve. Fresh crystals to form a bulk nearly equal to that of the whole fluid are added, and the dish is set in an incubator at 37° C. overnight. Next day a brown scum of precipitate will be found floating on the surface This contains the toxin. It is skimmed off with a spoon and placed in watch-glasses ; these are dried *in vacuo* and stored in the dark, also *in vacuo*, or in an exsiccator containing strong sulphuric acid For use the contents of one are dissolved up in a little normal saline solution.

The whole question of the parts played by toxins in bacterial action is manifestly very complex. On the one hand, we have a few diseases, for example, diphtheria and tetanus, in which

very characteristic effects are produced on special tissues by soluble toxins. On the other hand, we have the great mass of bacterial infections, in which the toxic effects are of a non-specific character, in the sense that they are not the result of an action on any particular tissue in the body, but on the vital processes of the organism as a whole. There is also the possibility that with any one species of organism different effects may be produced by, it may be, different elements in the protoplasm of the invading bacterial cell. Some of these elements may act on such specialised body cells as those of the nervous system, liver, or kidneys, giving rise to disturbances of metabolism. Other poisonous elements may act mainly on the defensive cells of the body, *e.g.* the leucocytes. A small dose of toxin may stimulate these cells to an activity which results in the infection being thrown off, either by the poison being neutralised, or by the supply of toxin being cut off by the killing of the bacterium producing it. A large dose of such a toxin may, on the other hand, altogether break down the defensive mechanism of the invaded body.

There is another point which must be kept in view, namely, that some of the phenomena which have been regarded as dependent upon the activity of bacterial toxins may possibly be related to the condition of anaphylaxis (p. 223). Anaphylaxis essentially consists in the development under certain circumstances in an animal of a hypersensitiveness to foreign albuminous materials which in themselves are not toxic. Effects of the gravest kind may be produced during this period of hypersensitiveness, and it has been thought that some of the phenomena of an infective disease, *e.g.* the intervention of an incubation period before symptoms occur, may be accounted for by the gradual development of hypersensitiveness to the proteins of the invading bacteria; and it has been demonstrated by methods to be referred to later, that in various infections the tissues of the patient are more susceptible to the products of the invading organisms than are the tissues of a normal individual. The phenomena of any bacterial disease may thus in reality be due to very different and complex causes.

The Nature of Toxins.—There is comparatively little known regarding this subject. The fact that many exotoxins are precipitated along with albumoses suggested the idea that they are formed from the medium in which the bacteria are growing by processes analogous to those of gastric digestion. Sidney Martin found that albumoses and peptones were formed by the action of certain pathogenic bacteria, and that the precipitate

containing these albumoses was toxic. A similar digestive action was found in the case of the tubercle bacillus by Kühne.

Further evidence that bacterial toxins are either albumoses or bodies having a still smaller molecule was adduced by C. J. Martin. By filling the pores of a Chamberland bougie with gelatin, he obtained what is practically a strongly supported colloid membrane through which dialysis can be made to take place under great pressure, say, of compressed oxygen. He found that in such an apparatus toxins—at least two kinds tried—will pass through just as an albumose will.

On the other hand, certain facts indicate that the exotoxins like the endotoxins are the product of internal metabolism in bacteria. Thus Brieger and Boer, working with bouillon cultures of diphtheria and tetanus, separated, by precipitation with zinc chloride, bodies which showed characteristic toxic properties, but which had the reactions neither of peptone, albumose, nor albuminate, and the nature of which is unknown. It has also been found that the bacteria of tubercle, tetanus, diphtheria, and cholera can produce toxins when growing in protein-free fluids. Further investigation is here required, for Uschinsky, applying Brieger and Boer's method to a toxin so produced, states that the toxic body is not precipitated by zinc salts, but remains free in the medium. If the toxins are really non-protein they may, on the one hand, be the final product of a digestive action—extra- or intra-cellular—or they may be the manifestation of a separate vital activity on the part of the bacteria. On the latter theory the toxicity of the toxic albumoses of Sidney Martin may be due to the precipitation of the true toxins along with these other bodies. From the chemical standpoint this is quite possible. Of the nature of the endotoxins nothing is known.

When we take into account the extraordinary potency of these poisons (in the case of tetanus the fatal dose of the pure poison for a guinea-pig must often be less than 0·000001 gram), we can understand how, altogether apart from their instability, attempts by present chemical methods to isolate them in a pure condition are not likely to be successful, and of their real nature we know nothing. Friedberger and Moreschi have shown that the intravenous injection in the human subject of a fraction of a loopful of a dead typhoid culture gives rise to toxic symptoms, including marked febrile reaction. Such injections are followed by the appearance of agglutinating and bacteriolytic substances in the serum. These results show that intracellular toxins may

be comparable with extracellular toxins so far as concerns the extremely small dose sufficient to produce toxic effects.

The comparison of the action of bacteria in the tissues in the production of these toxins to what takes place in the gastric digestion, has raised the question of the possibility of the elaboration by these bacteria of *ferments* by which the process may be started. Thus Sidney Martin put forward the view that ferments may be produced which we may look on as the primary toxic agents, and which act by digesting surrounding material and producing albumoses—these bodies being, as it were, secondary poisons. Hitherto all attempts at the isolation of bacterial ferments of such a nature have failed.

But apart from the fact that with such bacteria as those of tetanus and diphtheria, toxins may have a digestive origin, analogies have been drawn between ferment and toxic action. The chief facts upon which these have been founded are as follows The toxic products of these and other bacteria lose their toxicity by exposure to a temperature which puts an end to the activity of such an undoubted ferment as that of the gastric juice If diphtheria toxin be heated at 65° C. for one hour, it loses much of its toxic effect, and in the case of B. tetani all the toxicity is lost by exposure at this temperature. In regard to both diseases there is a still further fact which is adduced in favour of the toxic substances being of the nature of ferments, namely, the existence of a definite period of incubation between the injection of the toxic bodies and the appearance of symptoms This may be interpreted as showing that after the introduction of, say, a filtered bouillon culture, further chemical substances are formed in the body before the actual toxic effect is produced. Too much reliance must not be placed on such an argument, for in the case of tetanus, at least, the delay may be explained in part by the fact that the poison apparently has to travel up the nerve trunks before the real poisonous action is developed. Further, with some poisons presently to be mentioned which are closely allied to the bacterial toxins, an incubation period may not exist. It would not be prudent to dogmatise as to whether the toxins do or do not belong to such an ill-defined group of substances as the ferments. It may be pointed out, however, that the essential concept of a ferment is that of a body which can originate change without itself being changed, and no evidence has been adduced that toxins fulfil this condition. Another property of ferments is that so long as the products of fermentation are removed, the action of a given amount of ferment is indefinite. Again, in the case of toxins no evidence of such an occurrence has been found. A certain amount of a toxin is always associated with a given amount of disease effect, though a process of elimination of waste products must be all the time going on in the animal's body. Again, too much importance must not be attached to loss of toxicity by toxins at relatively low temperatures. This is not true of all toxins, and furthermore many proteins show a tendency to change at such temperatures ; for instance, if egg albumin be kept long enough at 55° C. nearly the whole of it will be coagulated. We must therefore maintain an open mind on this subject.

Similar Vegetable and Animal Poisons.—It has been found

that the bacterial poisons belong to a group of toxic bodies all presenting very similar properties, other members of which occur widely in the vegetable and animal kingdoms. Among plants the best known examples are the ricin and abrin poisons obtained by making watery emulsions of the seeds of the *Ricinus communis* and the *Abrus precatorius* (jequirity) respectively. From the *Robinia pseudacacia* another poison—robin—belonging to the same group is obtained. The chemical reactions of ricin and abrin correspond to those of the bacterial toxins. They are soluble in water, they are precipitable by alcohol, but being less easily dialysable than the albumoses they have been called toxalbumins. Their toxicity is seriously impaired by boiling, and they also gradually become less toxic on being kept. Both are among the most active poisons known—ricin being the more powerful. When they are injected subcutaneously a period of twenty-four hours usually elapses—whatever be the dose—before symptoms set in. Both tend to produce great inflammation at the seat of inoculation, which in the case of ricin may end in an acute necrosis ; in fatal cases hæmorrhagic enteritis and nephritis may be found. Both act as irritants to mucous membranes, abrin especially being capable of setting up most acute conjunctivitis. In the action of a poisonous fungus, *Amanita phalloides*, a similar toxin is at work. After an incubation period of some hours, symptoms of abdominal pain, diarrhœa with bloody stools, and, later, jaundice occur. *In vitro* the toxin has a hæmolytic action. As in the case of other poisons of this class, an antitoxin can be produced towards the fungus poison

It is also certain that the poisons of bees, of scorpions, and of poisonous snakes belong to the same group. The poisons derived from the last are usually called venins, and a very representative group of such venins derived from different species has been studied. To speak generally, there is derivable from the natural secretions of the poison glands a series of venins which have all the reactions of the bodies previously considered. Like ricin and abrin, they are not so easily dialysable as bacterial toxins, and therefore have also been classed as toxalbumins. Their properties are also similar ; many of them are destroyed by heat, but the degree necessary here also varies much, and some will stand boiling. There is also evidence that in a crude venin there may be several poisons differently sensitive to heat. All the venins are very powerful poisons, but here there is practically no period of incubation—the effects are almost immediate. An outstanding feature of the venins is the complexity of the crude poison secreted by any particular species of snake. C. J. Martin, in summing up the results of many observers, has pointed out that different venoms have been found to contain one or more of the following poisons : a neurotoxin acting on the respiratory centre ; a neurotoxin acting on the nerve endings in muscle ; a toxin causing hæmolysis ; toxins acting on other cells, *e.g.* the endothelium of blood vessels (this from its effects has been named hæmorrhagin), leucocytes, nerve-cells ; a toxin causing thrombosis ; a toxin having an opposite effect and preventing coagulation ; a toxin neutralising the bactericidal qualities of the body fluids and thus favouring putrefaction ; a toxin causing agglutination of the red blood corpuscles ; a proteolytic ferment ; a toxin causing systolic standstill of the excised heart. Any particular venom contains a mixture in varying proportions of such toxins,

and the different effects produced by the bites of different snakes largely depend on this variability of composition. The neurotoxic, the thrombotic, and the hæmolytic toxins are very important constituents of any venom. The toxicity of different venoms varies much, and no general statement can be made with regard to the toxicity of different poisons towards man. Lamb calculated that the fatal dose of crude cobra venom for man is probably about 0·015 of a gram, and that if such a snake bites with full glands many times this dose would probably be injected; but of course, the amount emitted depends largely on the period which has elapsed since the animal last emptied its glands When a dose of a venom not sufficient to cause immediate death from general effects is given, very rapid and widespread necrosis may often occur in a few hours round the site of inoculation.

An extremely important fact was discovered by Flexner and Noguchi, namely, that the hæmolytic toxin of cobra venom in certain cases has no action by itself on red corpuscles, but produces rapid solution when some normal serum is added, the latter containing a labile complement-like body, which activates the venom. In this there is a close analogy to what holds in the case of a hæmolytic serum deprived of complement by heat at 55° C (p. 198). So far no example of the activation of a bacterial toxin is known, but the results mentioned point to the possibility of this occurring in some cases in the tissues of the body.

There is another group of toxic manifestations which present some analogies to those of the bacterial toxins, but concerning which very little is known. The best example of these is found in the toxic properties of the serum of the eel. If a small quantity of such serum, say 0·25 of a c.c., be injected into a rabbit subcutaneously, death occurs in a few minutes. Although nothing is known of the substances giving rise to such effects, the phenomenon is to be considered as analogous to the action of bacterial toxins.

The Theory of Toxic Action.—While we know little of the chemical nature of any toxins, we may, from our knowledge of their properties, group together the tetanus, diphtheria, and botulismus poisons, ricin, abrin, snake poisons, and scorpion poisons. Besides the points of agreement already noted, all possess the further property that, as will be afterwards described, when introduced into the bodies of suitable animals they stimulate the production of neutralising substances called *antitoxins*; in other words, they act as *antigens*. The nature of the antagonism between toxin and antitoxin will be discussed later. Here, to explain what follows, it may be stated (1) that the molecule of toxin forms directly a combination with the molecule of antitoxin, and (2) that it has been shown that toxin molecules may lose much of their toxic power and still be capable of uniting with exactly the same proportion of antitoxin molecules. From these and other circumstances Ehrlich advanced the view that the toxin molecule has a complicated structure, and con-

tains two atom groups. One of these, the *haptophore* (ἅπτειν, to bind to), is that by which combination takes place with the antitoxin molecule, and also with presumably corresponding molecules naturally existing in the tissues. The other atom group he called the *toxophore*, and it is to this that the toxic effects are due. This atom group is brought into relation with the cell elements, *e.g.* the nerve cells in tetanus, by the haptophore group. Ehrlich explained the loss of toxicity which with time occurs in, say, diphtheria toxin, on the theory that the toxophore group undergoes disintegration. And if we suppose that the haptophore group remains unaffected we can then understand how a toxin may have its toxicity diminished and still require the same proportion of antitoxin molecules for its neutralisation. To the bodies whose toxophore atom groups have become degenerated, Ehrlich gave the name *toxoids*. His view with regard to the origin of toxoids from toxins has received ample confirmation within recent years by the work of Ramon and others, and is now generally accepted. The theory may afford an explanation of what has been suspected, namely, that in some instances toxins derived from different sources may be related to one another. For example, Ehrlich pointed out that ricin produces in a susceptible animal body an antitoxin which corresponds almost completely with that produced by another vegetable poison, robin (*vide supra*). This may be explained on the supposition that robin is a toxoid of ricin, *i.e.* their haptophore groups correspond, while their toxophore differ. The whole subject will be again referred to in the chapter on Immunity.

With regard to the intracellular toxins we shall see it is difficult to determine whether or not they share with the extracellular poisons the property of stimulating antitoxin formation, —if they do not, then they may belong to an entirely different class of substances. It is certain that immunity against such poisons is difficult to establish and is not of a lasting character. It may be said that Macfadyen, by grinding up typhoid bacilli frozen by liquid air, claimed that on thawing he obtained the endotoxins in liquid form, and he further stated that by using this fluid he could immunise animals not only against the toxins but also against the living bacteria. Other examples of similar nature might be quoted, but we may say that in the great majority of cases endotoxins, which, as has been said, are relatively heat-resistant, do not give rise to antitoxins on injection, that is, do not function as antigens. On the other hand, the injection of dead bacteria readily leads to the formation of

antibodies of other types—agglutinins, opsonins, etc. (p. 201). Vaughan considers that the poisonous protein split off by him from the bacterial substance is probably a polypeptid, and it is known that substances of this nature have not the antigenic property. If this poisonous protein is really the toxic agent in infections, we would thus have an explanation of the fact that in most instances antitoxins to endotoxins cannot be obtained. He considers that in the procedure followed by him the various secondary and tertiary groups are split off from the toxic molecule, and it is to these groups that the antigenic properties of the bacterial protoplasm, as shown by its giving rise to the other antibodies mentioned, are due. In any case, it seems pretty clear that the substances which give rise to the symptoms of general poisoning in infections do not act as antigens, apparently because they are of too simple a constitution or of too small a size.

We have already pointed out that those who claim for the aggressins a special character hold that the activity of these bodies has as its effect the interference with the phagocytic functions of the leucocytes. They also hold that a special type of immunity can be developed against the aggressins.

CHAPTER VI

IMMUNITY

Introductory.—By immunity is meant non-susceptibility to a given disease or to a given organism, either under natural conditions or under conditions experimentally produced. The term is also used in relation to the toxins of an organism. Immunity may be possessed by an animal naturally, and is then usually called *natural* immunity, or it may be *acquired* by an animal, either by its passing through an attack of the disease, or by means of artificial inoculation. It is to be noted that man and the lower animals may be exempt from certain diseases under natural conditions, and yet the causal organisms of these diseases may produce pathogenic effects when injected in sufficient quantity. Immunity is, in fact, of very varying degrees, and accordingly the use of the term has a relative significance. This is not only true of infection by bacteria, but of toxins also—when the resistance of an animal to these is of high degree, the resistance may in certain cases be overcome by a very large dose of the toxic agent. On the other hand, even in cases where the natural powers of resistance are high, these can be still further exalted by artificial means—that is, the natural immunity may be artificially intensified.

Acquired Immunity in the Human Subject.—The following facts are supplied by a study of the natural diseases which affect the human subject. First, in the case of certain diseases, one attack protects against another for many years, sometimes practically for a lifetime, *e.g.* smallpox, typhoid, scarlet fever, etc. Secondly, in the case of other diseases, *e.g.* erysipelas, influenza, and pneumonia, a patient may suffer from more than one attack. In the case of the diseases of the second group, however, experimental research has shown that in many of them immunity is developed, but is transient; and, though we cannot definitely state it as a universal law, it must be considered highly probable that the passing through an attack of an acute disease confers immunity for a longer or shorter period. The immunity is not, however, to be regarded as the result of the

disease *per se*, but of the bacterial products formed in the system during the disease. And, as will be shown below, by suitable gradation of the doses of such products, or by the use of weakened toxins, a high degree of immunity may be attained without the occurrence of any symptoms whatever. It has been found in the case of diphtheria, typhoid, cholera, pneumonia, etc., that in the course of the disease certain substances appear in the blood, which are antagonistic either to the toxin or to the vital activity of the organism. In such cases a process of immunisation would appear to be going on during the progress of the disease, and when this immunisation has reached a certain height, the disease naturally comes to an end. It cannot, however, be said as yet that such antagonistic substances are developed in all cases; although the results already obtained make this probable.

The facts known regarding vaccination and smallpox exemplify another principle, namely, that immunity may be conferred as the result of passing through a *modified* attack of a disease. We may take it as practically proved that vaccinia is variola or smallpox in the cow, and that when vaccination is performed, the patient is inoculated with a modified variola (*vide* p. 617). Vaccination produces certain pathogenic effects which are of trifling degree as compared with those of smallpox, and we find that the degree of protection is less complete and lasts a shorter time than that produced by the natural disease. Again, inoculation with lymph from a smallpox pustule produces a form of smallpox less severe than the natural disease but a much more severe condition than that produced by vaccination, and it is found that the degree of protection or immunity resulting occupies an intermediate position.

Artificial Immunity

Varieties.—According to the means by which it is produced, immunity may be said to be of two kinds, to which the terms *active* and *passive* are generally applied, or we may speak of immunity *directly*, or *indirectly*, *produced*.

Active immunity is obtained by (*a*) injections of the organisms either in an attenuated condition or in sub-lethal doses, or (*b*) by sub-lethal doses of their products, *i.e.* of their "toxins," the word being used in the widest sense. By repeated injections at suitable intervals the dose of organisms or of the products can be gradually increased; or, what practically amounts to the same, an organism of greater virulence or a toxin of greater

strength may be used. The establishment of immunity is attended by the appearance of *antibodies* in the serum, and the molecules of the bacteria or toxins which lead to the development of these are called *antigens*. Such methods constitute the means of *preventive inoculation* or *vaccination*. Immunity of this kind is comparatively slowly produced and lasts a considerable time, the duration varying in different cases. The principles of vaccination were originally extended by Wright to the *treatment* of disease.

Passive immunity depends upon the fact that if an animal be immunised to a very high degree by the previous method, its serum may have distinctly antagonistic or neutralising effects when injected into another animal along with the organisms, or with their products, as the case may be; that is, the antibodies developed by active immunisation may be transferred to a fresh animal. Such a serum, generally known as an *antiserum*, may exert its effects if introduced into an animal at the same time as infection occurs or even a short time afterwards; it can, therefore, be employed as a *curative* agent. The serum is also *preventive*, *i.e.* protects an animal from subsequent infection, but the immunity thus conferred lasts a comparatively short time. These facts form the basis of *serum therapeutics*. When such a serum has the power of neutralising a toxin it is called *antitoxic*; when, with little or no antitoxic power, it protects against the living bacterium in a virulent condition, it is called *antimicrobic* or *antibacterial* (*vide infra*).

In the accompanying table a sketch of the chief methods by which immunity may be artificially produced is given. It has been arranged merely for purposes of convenience and to aid subsequent description; the principles underlying all the methods are the same.

ARTIFICIAL IMMUNITY—METHODS

A. ACTIVE IMMUNITY—*i.e.* produced in an animal by an injection, or by a series of injections, of non-lethal doses of an organism or its toxins. It is essential, as a general rule, that the antigen should be introduced into the tissues, that is, "parenterally," and not into the alimentary tract.

1. *By injection of the living organisms.*

(*a*) Attenuated in various ways. Examples:

(1) By growing in the presence of oxygen, or in a current of air. (2) By passing through the

tissues of one species of animal (attenuation for another species). (3) By growing at abnormal temperatures, etc. (4) By growing in the presence of weak antiseptics, or by injecting the latter along with the organism, etc.

(*b*) In a virulent condition, in non-lethal doses.

2. *By injection of the dead organisms, which are sometimes "sensitised" by an antiserum.*
3. *By injection of filtered bacterial cultures,* i.e. *toxins; or of substances derived from such filtrates.*

These methods may also be combined in various ways.

B. PASSIVE IMMUNITY—*i.e.* produced in one animal by injection of the serum of another animal highly immunised by the methods of A.

1. *By antitoxic serum, i.e.* the serum of an animal highly immunised against a particular toxin.
2. *By antibacterial serum, i.e.* the serum of an animal highly immunised against a particular bacterium in the living and virulent condition.

Methods of producing Active Immunity

1. **By Living Cultures.**—(*a*) *Attenuated.*—In the earlier work on immunity in the case of anthrax, chicken cholera, swine plague, etc., the investigators had to deal with organisms of high virulence, which had accordingly to be reduced before the organisms could be injected in the living state. It is now found most convenient as a rule to start the process of active immunisation with the injection of dead cultures. The principle is the same as that of vaccination, and both attenuated cultures and also the dead cultures used for injection are often spoken of as *vaccines.* The virulence of an organism may be diminished in various ways, of which the following examples may be given:

(1) In the first place, practically every organism, when cultivated for some time outside the body, loses its virulence to a greater or less degree, and in the case of some this is very marked indeed, *e.g.* the pneumococcus. Pasteur found in the case of chicken cholera, that when cultures were kept for a time in ordinary conditions, they gradually lost their virulence, and that when subcultures were made the diminished virulence

persisted. Such attenuated cultures could be used for protective inoculation. He considered the loss of virulence to be due to the action of the oxygen of the air, as he found that in tubes sealed in the absence of oxygen the virulence was not lost. Haffkine attenuated cultures of the cholera spirillum by growing them in a current of air (p. 458).

(2) The virulence of an organism for a particular animal may be lessened by passing the organism through the body of another animal. Duguid and Burdon Sanderson found that the virulence of the anthrax bacillus for bovine animals was lessened by its being passed through guinea-pigs, the disease produced in the ox by inoculation from the guinea-pig being a non-fatal one. This discovery was confirmed by Greenfield, who showed that the bacilli cultivated from guinea-pigs preserved their property in cultures, and could therefore be used for protective inoculation of cattle. A similar principle was applied in the case of swine plague by Pasteur, who found that if the organism producing this disease was inoculated from rabbit to rabbit, its virulence was increased for rabbits but was diminished for pigs. The method of vaccination against smallpox depends upon the same principle (p. 615).

(3) Many organisms become diminished in virulence when grown at an abnormally high temperature. The method of Pasteur (p. 392), for producing immunity in sheep against anthrax bacilli, depends upon this fact. A virulent organism may also be attenuated by being exposed to an elevated temperature which is insufficient to kill it, as was found by Toussaint in the case of anthrax.

(4) Still another method may be mentioned, namely, the attenuation of the virulence by growing the organism in the presence of weak antiseptics. Chamberland and Roux, for example, succeeded in attenuating the anthrax bacillus by growing it in a medium containing carbolic acid in the proportion of 1 : 600.

These examples will serve to show the principles underlying attenuation of the virulence of an organism. There are, however, still other methods, most of which consist in growing the organism in conditions somewhat unfavourable to its growth, *e.g.* under compressed air, etc.

(*b*) *Immunity by living Virulent Cultures in Non-lethal Doses.*—Immunity may also be produced by employing virulent cultures in small, that is, non-lethal, doses. In subsequent inoculations the doses may be increased in amount. For example, immunity may thus be obtained in rabbits against the B. pyocyaneus.

Such a method, however, is difficult to carry out, and it has been found more convenient to commence the process of immunisation with dead or attenuated cultures, and then to continue with virulent cultures.

Exaltation of the Virulence.—The converse process to attenuation, *i.e.* the exaltation of the virulence, is obtained chiefly by the method of cultivating the organism from animal to animal—the method of *passage* discovered by Pasteur (first, we believe, in the case of an organism obtained from the saliva in hydrophobia, though having no causal relationship to that disease). This is most conveniently done by intraperitoneal injections, as there is less risk of contamination. The organisms in the peritoneal fluid may be used for the subsequent injection, or a culture may be made between each inoculation. The virulence of a great number of organisms can be increased in this way, the animals most frequently used being rabbits and guinea-pigs. This method can be applied to the organisms of typhoid, cholera, pneumonia, to streptococci and staphylococci, and in fact to those organisms generally which invade tissues. In many instances, however, it is difficult or even impossible to exalt the virulence of an organism by passage.

Depression Immunity.—A somewhat striking phenomenon in active immunisation is that described by Morgenroth, Biberstein, and Schnitzer. They found that when mice were the subjects of a chronic general streptococcic infection, the effects of an additional injection of virulent streptococci were diminished; and the important point is that such mitigation was manifest within twenty-four hours after the original inoculation. In some way the first injection had the effect of modifying or depressing the virulence of the organisms used in the second inoculation—hence the term applied by them. The nature of the phenomenon requires further elucidation.

2. Immunity by Dead Cultures of Bacteria.—In some cases a high degree of immunity against infection by a given microbe may be developed by repeated and gradually increasing doses of the dead cultures, the cultures being killed sometimes by heat, sometimes by exposure to some antiseptic. In this method the so-called endotoxins will be injected along with the other substances in the bacterial protoplasm, but the resulting immunity is chiefly directed against the vital activity of the organisms—is antibacterial rather than antitoxic (*vide infra*). The cultures when dead produce, of course, less effect than when living, and this method may be conveniently used in the initial stages of active immunisation—to be afterwards followed by

injections of the living cultures. The method is extensively used for experimental purposes, and is that adopted in anti-typhoid and anti-plague inoculations, and in the *treatment* of infections by means of vaccines. It is, however, not universally applicable.

Immunity by Sensitised Dead Cultures.—In this method, which was originally introduced by Besredka, the bacterial emulsion is treated with the corresponding antiserum, that is, the serum of an animal immunised against the particular bacterium, and after being left in contact for some time, the serum is separated by the centrifuge and the bacteria are thoroughly washed free of all traces of serum. The bacteria thus treated constitute the *vaccins sensibilisés*. It is claimed that, while immunity produced by them is rapidly developed and is of long duration, the local toxic effects on subcutaneous injection are very much lessened. The method has been applied in vaccination against typhoid, plague, cholera, and dysentery. Apparently in such sensitised vaccines the antigen molecules of the bacteria will be largely combined with anti-substances, and thus on theoretical grounds we would expect that only those molecules left free, or those which become free by dissociation, will be able to act as antigens and the antigenic power of the bacteria will be diminished. Certain observations show that this is the case, but it would be desirable to have fuller knowledge of the amounts of antibodies developed by the sensitised and non-sensitised bacteria respectively and of the relation of such amounts to the degree of protection afforded. It is possible that the slow and steady supply of free antigen by dissociation may specially favour the development of the antibody, while the antibody gives a degree of passive immunity.

Combination of Methods.—The above methods may be combined in various ways. By repeated injections of cultures at first in the dead condition, then in the living and attenuated state, and afterwards in the more virulent, and by increasing the doses, a high degree of immunity may be obtained. It is important, however, to note at this point that the most efficient methods of immunisation vary in the case of different infections, but that as a rule a more efficient immunity is developed by means of living than of dead cultures, as has been recently insisted on by Ledingham. It is doubtful whether a solid immunity against anthrax and the other virulent diseases investigated by Pasteur, can be developed otherwise than by means of attenuated living cultures. And in the case of others such as tuberculosis (Calmette), pneumococcus infections (Cecil and Blake), typhoid (Metchnikoff and Besredka), etc., living organisms are more effective than dead organisms as a vaccine. The most satisfactory method of preventive inoculation varies in different instances, and each disease must be individually investi-

gated. Further details will be given in connection with the special diseases.

3. Immunity by the Separated Bacterial Products or Toxins.—The organisms are grown in a fluid medium for a certain time, and the fluid is then filtered through a Chamberland or other porcelain filter. The filtrate contains the toxins, and it may be used unaltered, or may be concentrated in varying degrees by evaporation. It is important that the strain of organism used should be one which produces toxin abundantly; different strains vary greatly in this respect. The process of immunisation by the toxin is started by small non-lethal doses of the strong toxin, or by larger doses of toxin the power of which has been weakened by various methods (*vide infra*). Afterwards the doses are gradually increased. This method was carried out with a great degree of success in the case of diphtheria and tetanus. It appears capable of general application in the case of organisms where it is possible to get an active toxin from the filtered cultures. It was also applied in the case of snake venoms by Calmette and by Fraser, a high degree of immunity being produced.

The following may be mentioned as some of the most important examples of the practical application of the principles of active immunity, *i.e.* of protective inoculation: (1) Inoculation of sheep and oxen against anthrax (Pasteur) (p. 392); (2) Jennerian vaccination against smallpox (p. 618); (3) Anti-cholera inoculation (Haffkine) (p. 458); (4) Anti-plague inoculation (Haffkine) (p. 512); (5) Anti-typhoid inoculation (Wright and Semple) (p. 421); (6) Pasteur's method of inoculation against hydrophobia, which involves essentially the same principles (p. 629); (7) Toxin-antitoxin immunisation against diphtheria (p. 479) (in this case the antitoxin is combined with the toxin to prevent injurious effects).

Vaccines as a Method of Treatment.—Within recent times the principles of active immunity have been directly applied in the treatment of already existing disease. This is largely due to the work of Wright, who employed injections of dead cultures of the causal agents. The justification for such a procedure lies in his contention that in many cases infections are to be looked on as practically localised, *e.g.* the case of an acne pustule, or a boil; and that while the local capacities of resistance may have been lowered, resisting mechanisms in other parts of the body have not been brought into play. The vaccine may thus stimulate these, and the focus of bacterial growth may be flooded with antibacterial bodies.

It is generally recognised that, as a rule, the best results are obtained when an *autogenous* strain of the organism is used, that is, a strain cultivated from the lesion which is to be treated. (With regard to the details of the preparation of the vaccines, see p. 139 ; the general principles supposed to underlie their use will be discussed in connection with tuberculosis, p. 338.) Vaccines have been used extensively in the treatment of acne, boils, sycosis, tuberculosis, infections of the genito-urinary tract by the B. coli, infections of joints by the gonococcus, and in many cases considerable success has followed the treatment. Favourable results have also been recorded in the case of more general infections, such as ulcerative endocarditis, septicæmia, typhoid fever, etc. In such cases it is stated that the best results are obtained from the use of sensitised vaccines (*vide supra*).

Sensitised vaccines are prepared by subjecting living cultures (preferably of autogenous strains) to the action of say 5 c.c. of the appropriate antiserum for three hours at 37° C. The sensitised bacteria are deposited by centrifuging, emulsified in saline containing 0·5 per cent. phenol, and again kept at 37° C. for three hours, so that the phenol may kill them. The vaccine is then ready for use. In infections with streptococci or the B. coli from ten to forty millions may be given, the dose being repeated in twenty-four hours. In very acute infections a few hundred thousand sensitised bacteria may produce definite results, and if improvement of symptoms occurs the dose may be cautiously repeated in six hours.

Active Immunity by Feeding.—Ehrlich found that mice could be gradually immunised against ricin and abrin by feeding them with increasing quantities of these substances (*vide* p. 171). In the course of some weeks' treatment in this way the resulting immunity was of so high a degree that the animals could tolerate on subcutaneous inoculation 400 times the dose originally fatal. Fraser also found in the case of snake venom that rabbits could, by being fed with the poison, be immunised against several times the lethal dose of venom injected into the tissues. In such cases some of the molecules which act as antigens apparently pass through the intestinal wall unchanged.

Local Immunity.—The fact that in the case of erysipelas the infection may be dying out at one place while it is spreading at another, indicates that in the former immunity has been in some way established. Besredka has in recent years published a series of papers which bear on this subject. He has found that killed cultures of streptococci do not confer immunity when injected subcutaneously but do so when injected into the skin, the larger the number of points of injection the higher being the immunity. Filtrates of cultures are also effective ;

they contain an "anti-virus" which produces immunity only when injected into the skin or applied over the surface. A somewhat similar example of local immunity was observed in the case of infection through the skin in anthrax (p. 395). He finds also that it is possible to produce a local immunity of the respiratory tract against B. diphtheriæ by intratracheal injection of the organism in the dead condition; and of the intestine against B. paratyphosus by previous oral administration of ox bile with the living organisms. In all these cases, immunity does not depend on the presence of antibodies in the blood. They are examples of what he calls the "autonomy" of organs. The subject is one of much importance, but further research is necessary.

Active immunity of high degree developed by the methods described may be regarded as *specific*, in the sense explained below. A certain degree of immunity, or rather of increased general resistance of parts of the body (for example, the peritoneum), can, however, be produced by the injection of various substances—bouillon, blood serum, solution of nuclein, etc. (Issaeff). These agents probably act by producing a local leucocytosis.

The Properties of the Sera of Highly Immunised Animals

Antibodies and their Specificity.—The fundamental fact in passive immunity, namely, that immunity can be transferred to another animal, shows that the serum in question differs from the serum of a normal animal in containing antagonistic substances to the toxin or bacterium as the case may be—these being generally spoken of as antibodies. The development of these bodies, first observed in the case of the injection of toxins, is found to occur when a great many different substances are introduced into the tissues of the living body. We can, in fact, divide organic molecules into two classes—(*a*) those which give rise to the production of antibodies, and are thus known as *antigens*, and (*b*) those which have not this property. Amongst the former are various toxins, molecules of tissue cells, bacteria, red corpuscles, soluble tissue constituents of animal or vegetable origin, etc. They are all probably of protein nature, though their true constitution is not known, and none of them have been obtained in a pure condition. Amongst the substances which do not act as antigens may be placed the various poisons

of known constitution, glucosides, alkaloids, etc. Numerous attempts have been made to obtain anti-substances by the injection of lipoids, but though success has been claimed by some observers it may be stated that up to the present we have no satisfactory evidence that lipoids free from protein possess the antigenic function. Proteins in process of hydrolysis rapidly lose their antigenic function, amino-acids and probably also peptones being devoid of the property. We may also state at present that the antibody forms a chemical or physical union with the particular antigen which has led to its development, and we shall discuss the evidence for this later. Furthermore, the antibody has apparently a specific combining group which fits, as it were, a group in the corresponding antigen, the two groups having been compared to a lock and key. It is, however, to be noted that this specificity is a chemical or physical one rather than a biological one. An antiserum, for example, developed by the injection of bacterium A may also have some effect on bacterium B, and thus appear not to be specific. It is known, however, that the antigens in bacterium A are not all identical, and that some of them may be present, though in smaller proportion, in bacterium B; thus the theory of combining specificity is not invalidated. The number of different anti-substances, as judged by their combining properties, would appear to be almost unlimited, a fact which throws new light on the complexity of the structure of living matter.

In connection with the question of *specificity* the work of Obermayer and Pick and of Landsteiner on modification of the antigenic function of protein by chemical means is of great importance. This has been effected by introducing new groups such as iodine, azo- and nitro-radicals, by acetylation or methylation of proteins, etc. Each modified protein acts as a specific antigen and gives rise to a corresponding antibody even in the animal from which the protein was originally derived. For example, Landsteiner and Jablons found that rabbits injected with acetylated horse serum develop antibodies which react specifically with acetylated serum-protein from various other animals (hen, rabbit) in addition to the horse, but not with normal horse's serum or with diazo- or nitro-serum protein. Rabbits also produce an antiserum to acetylated rabbit serum. The process of acetylation has thus deprived the serum of its original species specificity and has given it a new specificity which may be called *structure specificity*. These results have an important bearing on Ehrlich's view regarding the mode of

formation of antibodies under the influence of antigens, as will be discussed later. The alteration of proteins by physical means such as boiling leads to rather different effects. Boiled protein gives rise to antibodies which react with boiled protein, but only when the latter belongs to the species from which the antigen was obtained; in this case the species specificity is preserved.

When anti-substances are studied as regards their action *in vivo* or *in vitro* on the substances with which they combine, it is found that (*a*) in certain cases simple combination may occur (*e.g.* antitoxic action), (*b*) in other cases physical effects may be associated with combination (*e.g.* agglutination or precipitation), and (*c*) in a third group of cases the antibody may lead to the union of another body normally present in serum, called complement or alexin. In this third group the combination of complement may or may not result in physical change in the antigen, the evidence of the latter occurrence being elicited by the fixation of complement method (p. 134). Antibodies of the third class are known as immune-bodies or amboceptors (Ehrlich) or sensitising substances—*substances sensibilisatrices* of French writers.

Such are the three classes of antibodies usually recognised, but while the classification is convenient they must not be regarded as quite distinct. It has been recently observed, for instance, that the combination of toxin and antitoxin may be attended by a physical change, namely, flocculation, and it is also known that the antibody which acts as a precipitin is closely related to, if not identical with, the antibody which acts as an immune-body and leads to the fixation of complement.

It is to be noted, however, that the presence of antibodies in the blood is not essential to immunity, since after they have disappeared the animal may still possess immunity. There is thus some change effected in the cells of the body which results in protection, but its nature is not fully known. It has been found, however, that an immunised animal without antibodies in the blood reacts in a different way from the normal animal, inasmuch as antibodies are more rapidly formed on reintroduction of antigen. But whether this altered power of antibody production is the full explanation of the immunity, is still doubtful. It is certain that in some cases the immunity resides locally in the tissues, *e.g.* in the skin when immunity to erysipelas has been established (p. 183).

After this preliminary statement in explanation, we shall

consider the actual properties of the two classes of serum, and later we shall resume the theoretical consideration.

Antitoxic Serum.—In a previous chapter (p. 164) a distinction has been drawn between exo- and endo-toxins, and with regard to these the general statement may be made that while antitoxins are, as a rule, comparatively easily obtained in the case of the former, the matter is quite otherwise in the case of the latter. In fact some writers have gone so far as to say that antitoxins to endotoxins cannot be obtained. Such an extreme view seems hardly justifiable, since it has been claimed that antitoxins can be developed to the endotoxins of the cholera vibrio and other organisms. Nevertheless we have the important fact that in many cases by the injection of dead cultures an active antibacterial serum can be obtained which has no neutralising action on the endotoxins, and we must conclude that many endotoxins do not act as antigens (p. 164). The best examples of antitoxic sera are those of diphtheria, tetanus, and botulismus, though similar principles and methods are involved in the case of the antisera to ricin and abrin, and to snake poison. We shall here speak of diphtheria and tetanus. The steps in the process of preparation may be said to be the following: first, the preparation of a powerful toxin; second, the estimation of the power of the toxin; third, the development of antitoxin in the blood of a suitable animal, by gradually increasing doses of the toxin; fourth, the estimation from time to time of the antitoxic power of the serum of the animal thus treated.

1. *Preparation of the Toxin.*—The mode of preparation and the conditions affecting the development of diphtheria and tetanus toxins are described later (pp. 473, 533). The culture of the organism is filtered through a Chamberland or similar filter when the maximum degree of toxicity has been reached. The term "toxin" is usually applied for convenience to the bacterium-free filtrate.

2. *Estimation of the Toxin.*—The power of the toxin is estimated by the subcutaneous injection of varying amounts in a number of guinea-pigs, and the minimum dose which will produce death is thus obtained. This, of course, varies in proportion to the weight of the animal, and is expressed accordingly. In the case of diphtheria, in Ehrlich's standard, the minimum lethal dose—known as M.L.D.—is the smallest amount which will certainly cause death in a guinea-pig of 250 grams within four days. The testing of a toxin directly is a tedious process, and in actual practice, where many toxins have to be

dealt with, it is found more convenient to test them by finding how much will be neutralised by a certain amount of a standard antitoxic serum, namely, an "immunity unit" (p. 189).

3. *Development of Antitoxin.*—The earlier experiments on tetanus and diphtheria were performed on small animals, such as guinea-pigs, but afterwards the sheep and the goat were used, and finally horses. In the case of the small animals it was found advisable to use in the first stages of the process either a weak toxin or a powerful toxin modified by certain methods. Such methods are the addition to the toxin of terchloride of iodine (Behring and Kitasato), the addition of Gram's iodine solution in the proportion of one to three (Roux and Vaillard), and the plan, adopted by Vaillard in the case of tetanus, of using a series of toxins weakened to varying degrees by being exposed to different temperatures, namely, 60°, and 55°, and 50° C. In recent years formalin (in weak solution) has been used by Glenny, Ramon, and others, in the early stages of immunisation. It diminishes the toxicity of the toxin without appreciably affecting its antigenic property, apparently by leading to the formation of toxoid (p. 173). In the case of large animals immunisation is sometimes started with small doses of unaltered toxin; and the doses are gradually increased. It has long been recognised that horses vary greatly in their resistance to diphtheria toxin, and also that some of them have a certain amount of antitoxin in their blood under natural conditions. Glenny has found that such animals produce toxin rapidly, whilst those with no natural immunity do not respond readily to immunisation. The production of diphtheria antitoxin occurs most satisfactorily when the toxin is injected subcutaneously or intramuscularly; when the intravenous route is used antitoxin formation is deficient or may be almost nil (Madsen). The antitoxin content of the serum is estimated from time to time, the object being, of course, to raise it to as high a figure as possible. It is found that each injection produces a certain amount of fall in the antitoxin value—the so-called "negative phase"—and this, in favourable cases, is followed by a rise to a higher level than before. (Similar phenomena are observed in the development of all other classes of antibodies.) In all cases of immunising the general health of the animal ought not to suffer. If the process is pushed too rapidly the antitoxic power of the serum may diminish instead of increasing, and a condition of marasmus may set in and may even lead to the death of the animal. After a sufficiently high degree of antitoxic power has been reached, the

animal is bled under aseptic precautions, and the serum is allowed to separate in the usual manner. It is then ready for use, but some weak antiseptic, such as 0·5 per cent. carbolic acid, is usually added as a preservative. Other antitoxic sera are prepared in a corresponding manner. Some further facts about antitetanic serum are given on p. 533.

4. *Estimating the Antitoxic Power of, or "Standardising," the Serum.*—This is done by testing the effect of various quantities of the serum of the immunised animal against a certain amount of toxin. Various standards have been used, of which the two chief are those of Ehrlich and of Roux. Ehrlich adopted as the *immunity unit* the amount of antitoxic serum which neutralises 100 times the minimum lethal dose of a given specimen of toxin, serum and toxin being mixed together, diluted up to 4 c.c., and injected subcutaneously into a guinea-pig of 250 grams weight, the prevention of the death of the animal for four days being taken as the indication of neutralisation. One c.c. of a serum, of which 0·02 c.c. will protect against 100 times the lethal dose, will possess 50 immunity units. Sera have been prepared of which 1 c.c. has the value of 800 units or even more. As a standard in testing, Ehrlich employed quantities of serum of known antitoxic power in a dry condition, preserved in a vacuum in a cool place, and in the absence of light. With such a standard test-serum any newly prepared serum can readily be compared.

An interesting physical change resulting from the union of toxin and antitoxin is the flocculation recently discovered by Ramon in the case of diphtheria toxin. He found that when graded doses of toxin are added to a unit of antitoxin, flocculation occurs in some of the tubes, and that the tube in which this first appears is the one in which neutralisation is complete, that is, the one in which there is excess neither of toxin or antitoxin. This flocculation occurs at room temperature, more rapidly at higher temperatures, but in estimating its first appearance it is preferable to work at the temperature of the laboratory. Ramon's results have been confirmed by Glennie and others, and the phenomenon has come to be of service in standardising diphtheria antitoxin, as it supplies a method much more rapid and more easily carried out than any previously in use.

Roux adopted a standard which represents the animal weight in grams protected by 1 c.c. of serum against the dose of virulent bacilli lethal to a control guinea-pig in thirty hours, the serum being injected twelve hours previously. Thus, if 0·01 c.c. of a

serum will protect a guinea-pig of 500 grams against the lethal dose, 1 c.c. (1 gram) will protect 50,000 grams of guinea-pig, and the value of the serum will be 50,000.

Sera of Animals immunised against Vegetable and Animal Poisons.—It was found by Ehrlich in the case of the vegetable toxins, ricin, and abrin, and also by Calmette and Fraser in the case of the snake poisons, that the serum of animals immunised against these respective substances had a protective effect when injected along with them into other animals. Ehrlich found, for example, that the serum of a mouse which had been highly immunised against ricin by feeding as described above, could protect another mouse against forty times the fatal dose of that substance. He considered that in the case of the two poisons, antagonistic substances—"anti-ricin" and "anti-abrin"—were developed in the blood of the highly immunised animals. A corresponding antagonistic body, to which Fraser gave the name "antivenin" appears in the blood of animals in the process of immunisation against snake poison.

These investigations are specially instructive, as such vegetable and animal poisons, both as regards their local action and the general toxic phenomena produced by them, present, as we have seen, an analogy to various toxins of bacteria.

Nature of Antitoxic Action.—This subject is only part of the general question with regard to the relation of antibodies to their corresponding antigens, but it is with regard to antitoxic action that most of the work has been done. We have to consider here two points, namely, (*a*) the relation of antitoxin to toxin, and (*b*) the source of the antitoxin. With regard to the former subject there is now no doubt that the antagonism between toxin and antitoxin is not a physiological one, but that the two bodies unite *in vitro* to form a compound inert towards the living tissues, there being in the toxin molecule an atom group which has a specific affinity for the antitoxin molecule or part of it.

When toxin and antitoxin are brought together *in vitro*, it can be proved that their behaviour towards each other resembles what is observed in physico-chemical reactions. Thus it has been found that a definite period of time elapses before the neutralisation of the toxin is complete, that neutralisation takes place more rapidly in strong solutions than in weak, and that it is hastened by warmth and delayed by cold. C. J. Martin and Cherry, and also Brodie, showed that in the case of the toxin of diphtheria and in that of an Australian snake poison, the toxin molecules will pass through a colloid membrane (p. 169), whilst those of the corresponding antitoxin will not. Now, if a mixture of equivalent parts of toxin and antitoxin is freshly prepared and at once filtered, a certain amount of toxin will pass through, but the longer such a mixture is allowed to stand before filtration the

less toxin passes, till a time is reached when no toxin is found in the filtrate. Further, if the portion of fluid which at this stage has not passed through the filter be injected into an animal, no symptoms take place. Again, in cases where the toxin has some definite physical effect, demonstrable *in vitro*, *e.g.* lysis, agglutination, coagulation, or the prevention of coagulation, its action can be annulled by the antitoxin; in such circumstances manifestly no physiological action of antitoxin through the medium of the cells of the body can come into play. The flocculation resulting from the interaction of toxin and antitoxin is another clear indication of physico-chemical combination.

Although authorities are now agreed as to the direct combination of toxin and antitoxin, there is still much uncertainty as to the exact nature of this union. Regarding this subject there may be said to be three chief views—(*a*) that of Ehrlich, according to which there is a firm chemical union of toxin and antitoxin, and the former is not homogeneous but consists of different molecules; (*b*) that of Arrhenius and Madsen, who consider that the phenomena correspond to the behaviour of two substances in weak chemical union; and (*c*) that of Bordet, who regards the combination to be not of chemical, but of physical nature, corresponding to a process of adsorption.

Controversy on this question may be said to date from the important work of Ehrlich on the neutralisation of diphtheria toxin. Using an immunity unit of antitoxin (the equivalent of 100 doses of toxin) he determined with any example of crude toxin the largest amount of toxin which could be neutralised completely, so that no symptoms resulted from an injection of the mixture. This amount he called the *limes null* dose, expressed as L_o. He then investigated the effects of adding larger amounts of toxin to the immunity unit and observed the quantity which was just sufficient to produce a fatal result, that is, which contained one M.L.D. of free toxin; this amount he called the *limes todtlich*, fatal limit, expressed as L_t. Now if, as he supposed, the union of toxin and antitoxin resembled that of a strong acid and base, L_t–L_o ought to be the equivalent of a minimum lethal dose of the toxin alone. This, however, was never found to be the case, the difference being always considerably more than one M.L.D. This, in brief, is what is known as the "Ehrlich phenomenon," and it has been explained by him as the result of the presence of toxoids (*vide* p. 173), *i.e.* toxin molecules in which the toxophore group has become degenerated. He distinguishes three possible varieties of such bodies according to the affinity of the haptophore group, namely, *prototoxoid* with more powerful affinity than the toxin molecule, *epitoxoid* with less powerful affinity, and *syntoxoid* with equal affinity. The presence of epitoxoids would manifestly explain the above phenomenon. The L_o dose would represent toxin+epitoxoid molecules all united to anti-

toxin molecules, and the presence of another M.L.D. of toxin would not result in there being a free fatal dose, but in the excess of toxin taking the place of epitoxoid. Several lethal doses would need to be added before the mixture was sufficient to produce a fatal result —that is, $L_t - L_o$ would equal several M.L D.'s.

The main contention of Madsen and Arrhenius is that the toxin-antitoxin combination is not a firm one but a reversible one, and is governed by the laws of physical chemistry. For example, in the case of a mixture of ammonia and boric acid (*i.e.* of a weak base and a weak acid) in solution, there is a constant relation between the amounts of each of the substances in the free condition and the amounts in combination—the combination is reversible, so that if some of the free ammonia were removed a certain amount of the combined ammonia would become dissociated to take its place; further, if to the mixture, in a state of equilibrium, more ammonia or more boric acid were added, part would remain free while part would combine. Accordingly, if toxin and antitoxin behaved in a similar manner, an explanation of the Ehrlich phenomenon would be afforded. Madsen and Arrhenius worked out the question in the case of a great many toxins, and found that the graphic representation of neutralisation is in every case a curve which can be represented by a formula.

According to Bordet's view, the union of toxin-antitoxin does not correspond to that which takes place in ordinary chemical union, but is a physical interaction of bodies in a colloidal state, the action being one of the so-called adsorption phenomena. The smaller toxin molecule becomes entangled as it were in the larger antitoxin one, very much as a dye becomes attached to the structure of a thread. He considers also that there is no definite quantitative relationship in the combination of the molecules of the two substances, different amounts of antitoxin being spread over, as it were, and affecting in varying degree, all the molecules of a given amount of toxin.

It should be noted in connection with this controversy that there are two questions which may be independent of each other, namely: (1) Does the "toxin" in any particular case represent a single substance or several? (2) What is the nature of the combination of any one constituent substance and its antibody —is it reversible or is it not? It seems impossible to explain the facts with regard to diphtheria toxin on the hypothesis of a single substance, even if this should have its combining and toxic actions equally weakened; "toxoids" in Ehrlich's sense must, in our opinion, be supposed. His results with regard to the existence of toxoids (epitoxoids at least) have received confirmation from recent workers. Ramon found that if diphtheria toxin was treated with 0·3 to 0·4 per cent. formalin, it lost its toxic action, but still retained its antigenic property and also gave the flocculation reaction as before. A similar change gradually occurs in a toxin when it is kept in the incubator for

a prolonged period. He called this detoxicated toxin "anatoxin," but it seems simply to correspond with Ehrlich's toxoids. Glenny and his co-workers confirm the view as to the existence of toxoid; they find that antitoxin has a greater affinity for toxin than for toxoid and may dissociate from combination with either. Then there is an important fact established by Danysz and by v. Dungern, namely, that the amount of toxin neutralisable by a given amount of antitoxin is different according as the toxin is added in several moieties or all at once—in the latter case the amount of toxin neutralisable is greater. There seems no explanation of this according to the view of Madsen and Arrhenius, as the same state of equilibrium ought to be reached in the two cases—that is, the amounts of toxin neutralised should be the same. There are many facts which are in conformity with Bordet's view. We have undoubtedly to deal with the combination of two colloids, and the flocculation which may be observed (p. 189) corresponds to a well-known phenomenon in the interaction of colloids. The chief difficulty arises in connection with specificity, as this would appear to depend upon the chemical structure and especially the arrangement of radicals, and thus to rest on a chemical rather than a physical basis.

An important factor in the union of toxin and antitoxin is the time necessary for the union to be complete. Morgenroth has shown that in the case of diphtheria toxin this is considerable—about twenty-four hours. Up to this time, mixtures of toxin and antitoxin, when injected intravenously, show decreasing degrees of toxicity according to the time they have been kept. On the other hand, when the subcutaneous method of injection is used the time interval has no effect, and this he considers to be due to a catalytic action of the tissues which accelerates the union of the two substances. He found also that the combination toxin-antitoxin could be broken up by dilute hydrochloric acid and the two constituents recovered; the union is thus reversible. Sachs obtained similar results by means of alkali. A striking phenomenon, which apparently points to the reversibility of the combination, was noted by Behring in the case of diphtheria toxin, and afterwards studied by Madsen and by Otto and Sachs in the case of botulismus toxin, namely, that when a certain amount of a mixture of toxin and antitoxin was found to be neutral on injection, a fraction of this amount might produce toxic phenomena or even death. This was apparently due to dissociation of the toxin in the greater dilution, and in favour of this being the case Otto and Sachs found that when the mixture was allowed to stand for twenty-four hours, so that combination was complete, the phenomenon no longer occurred. Other facts might be brought forward which show that the firmness of union of toxin and antitoxin increases with time, or in other words, that dissociation becomes more difficult. There is little

doubt that there are varying degrees of firmness of union of an antigen and its antibody, and varying periods necessary for the combination to become complete. It was shown by Morgenroth, and by Muir independently, that the union of a hæmolytic immune-body with the corresponding red corpuscle was of reversible nature; the latter observer found, however, that in this case the union was not increased in firmness after twenty-four hours.

A statement on the general question is at present impossible; we can only say that *direct combination of the two bodies does occur; that sometimes, probably often, the "toxin" contains different toxic bodies with varying affinity; and that in certain instances the combination has been proved to be reversible, but to what extent this is generally true remains still to be determined. In all cases the outstanding feature is the specific nature of the combination, and of this no satisfactory explanation can as yet be given.*

Antibacterial Serum.—The stages in the preparation of antibacterial sera correspond to those in the case of antitoxic sera, but living, or, in the early stages, dead cultures are used instead of toxin separated by filtration, and in order to obtain a serum of high antibacterial power it may ultimately be necessary to use a very virulent culture in large doses. For this purpose a fairly virulent culture is obtained fresh from a case of the particular disease, and its virulence may be further increased by the method of *passage*. This method of obtaining a high degree of immunity against the microbe is specially applicable in the case of those organisms which invade the tissues and multiply to a great extent within the body, and of which the toxic effects, though always existent, are proportionately small in relation to the number of organisms present.

The important result obtained by such experiments is, that if an animal be highly immunised by the method mentioned, the development of the immunity is accompanied by the appearance in the blood of *protective* substances, which can be transferred to another animal. The law enunciated by Behring regarding immunity against toxins thus holds good in the case of the living organisms, as was first shown by Pfeiffer. The latter found, for example, that in the case of the cholera organisms, so high a degree of immunity could be produced in the guinea-pig, that 0·002 c.c. of its serum would protect another guinea-pig against ten times the lethal dose of the organisms, when injected along with them. Here again is presented the remarkable potency of the antagonising substances in the serum, which in this case lead to the destruction of the corresponding microbe.

The presence of antibodies in the blood in such actively immunised animals is, of course, a matter of fundamental importance. But it must not be inferred that all the phenomena can thus be explained. For instance, an antiserum to the anthrax bacillus can be developed and by means of it passive immunity can be transferred to another animal; but the preventive property of the serum cannot be explained by the antibodies which can be demonstrated *in vitro*. Similarly, the highly protective effect of an anti-pneumococcus serum cannot at present be satisfactorily correlated with its content in antibodies.

The *anti-streptococcic serum* of Marmorek may be briefly described, as it has come into extensive practical use. This observer found that he could intensify the virulence of a streptococcus by growing it alternately in the peritoneal cavity of a guinea-pig and in a mixture of human blood serum and bouillon (*vide* p. 248). The virulence became so enormously increased by this method, that when only one or two organisms were introduced into the tissues of a rabbit a rapidly fatal septicæmia was produced. Streptococci of this high degree of virulence were used first by subcutaneous, afterwards by intravenous injection, to develop a high degree of resistance in the horse. Injections were continued over a considerable period of time, and the protective power of the serum was tested by mixing it with a certain dose of the virulent organisms, and then injecting into a rabbit. The serum of a horse highly immunised in this way constitutes the anti-streptococcic serum which has been extensively used in many cases of streptococcic invasion in the human subject. Marmorek, however, found that this serum had little antitoxic power—that is, could only protect from a comparatively small dose of toxin obtained by filtration of cultures.

Anti-typhoid, anti-cholera, anti-pneumococcic, anti-meningococcic, anti-plague, and other sera are all prepared in an analogous manner.

Properties of Antibacterial Serum.—We have here to consider the three main actions mentioned above, namely, (*a*) bactericidal and lysogenic or bacteriolytic action, (*b*) opsonic action, and (*c*) agglutination and the closely allied precipitation. Of these the two first are those chiefly concerned with the protective property of an antibacterial serum. These various properties are due to the presence of corresponding antibodies in the serum—immune-bodies, opsonins, agglutinins, etc.—but, as already stated, it is not to be assumed that these are always separate and distinct substances; they are in fact recognisable only by their effects.

(*a*) *Bactericidal and Bacteriolytic Action.*—Pfeiffer found that if certain organisms, *e.g.* the cholera spirillum, were injected into the peritoneal cavity of a guinea-pig highly immunised

against these organisms, they lost their motility almost immediately, gradually became granular, swollen, and then disappeared in the fluid—these changes constitute what is now generally known as " Pfeiffer's phenomenon " or bacteriolysis. It was subsequently shown, however, by Metchnikoff and by Bordet that bacteriolysis might occur outside the body by the addition of fresh peritoneal fluid or normal serum to the heated immune-serum. Pfeiffer also found that an antiserum heated to 70° C. for an hour produced the reaction when injected with the corresponding organisms into the peritoneum of a fresh animal. The outcome of these and subsequent researches was to show that when an animal is immunised against a bacterium, there appears in its serum an antibody, which is generally known as *immune-body*, *amboceptor* (Ehrlich), or *substance sensibilisatrice* (Bordet); it is comparatively stable, resisting usually a temperature of 65° C. for an hour. It cannot produce the destructive effect alone, but requires the addition of a substance normally present in the serum, which is spoken of under various names—*complement* (Ehrlich), *alexin* or *cytase* (French writers). The complement is relatively unstable, being rapidly destroyed by a temperature of 60° C., and it is not increased in amount during the process of immunisation. Though ferment-like in its instability, it differs from a ferment in being fixed or used up in definite quantities.

Observation has shown that complement is not a single substance, but is really made up of several components. Ferrata, who was the first to establish this fact, employed the following method: Fresh guinea-pig's serum is dialysed against running water for twenty-four hours; the precipitate which has formed at the end of that time is separated by the centrifuge, washed several times in distilled water, and then dissolved in normal salt solution. The separated fluid is passed through thick filter paper. The component in the solution of the precipitate unites directly with sensitised corpuscles—and then that in the separated fluid enters into combination; hence they have been called by Brand " middle-piece " and " end-piece " respectively. The separation by such a method is, however, far from being a complete one. The method of Liefmann, which is the most satisfactory, is the following: The serum is diluted by the addition of 9 volumes of distilled water, and then carbonic acid gas is passed through till globulin is precipitated. The precipitate is separated off by the centrifuge, and the clear fluid contains the end-piece, diluted, of course, ten times: The precipitate, containing the mid-piece, is dissolved in 0·8 per cent. sodium chloride solution, a convenient amount being twice the volume of the original serum. During the process of preparation, and afterwards, the serum and the diluting fluids ought to be chilled to a temperature a little above 0° C.; the serum should also

be used as fresh as possible after the blood is withdrawn from the body. The work of Browning and Mackie who fractioned complement-containing serum with ammonium sulphate as well as by Liefmann's procedure, has shown that the constitution of complement is even more complex than the above results would indicate.

The phenomenon of bacteriolysis is, however, only seen in the case of certain organisms when an animal is highly immunised against them; the typhoid and cholera group are outstanding examples. It is also to be noted that it sometimes is seen in the case of a normal serum (*vide* Natural Immunity). In other cases the bactericidal effect of a serum may occur without lysis of the bacteria, though other structural changes may be produced. In still other instances, *e.g.* the antisera to staphylococci, streptococci, plague bacilli, etc., a bactericidal effect may be wanting; nevertheless it may be shown that an immune-body is developed in the process of immunisation. This may be done by observing the increased amount of complement which is fixed through the medium of the antiserum (immune-body), sensitised red corpuscles being used as the test for the presence of free complement. The method is described on p. 134.

The all-important action of the immune-body is thus to bring an increased amount of complement into union with bacteria; whether death of the bacteria will result or not will depend ultimately on their sensitiveness to the action of the particular complement.

It is to be noted that in the case of a bactericidal serum there is an optimum amount of immune-body which gives the greatest bactericidal effect with a given amount of complement. If this amount of immune-body be exceeded, the bactericidal action becomes diminished and may be practically annulled. This result, which is generally known as the "Neisser-Wechsberg phenomenon," has been the subject of much controversy, and cannot yet be said to be satisfactorily explained; it is apparently of the nature of a "zone phenomenon" (cf. p. 204). (Regarding some theoretical considerations as to the therapeutic applications of antibacterial sera, *vide* p. 212.)

The laws of lysogenesis are, however, not peculiar to the case of solution of bacteria by the fluids of the body, but hold also in the case of other organised substances, red corpuscles, leucocytes, etc., when these are introduced into the tissues of an animal as in a process of immunisation. Of such sera the hæmolytic have been most fully studied, and, owing to the delicacy of the reaction and the ease with which it can be observed, have been the means of throwing much light on the

process of lysogenesis, and thus on one part of the subject of immunity. A short account of their properties may now be given.

Hæmolytic and other Sera.—It has long been known that in some instances the blood serum of one animal has, in a certain degree, the power of dissolving the red corpuscles of another animal of different species; in other instances, however, this property cannot be detected. Bordet showed that if one animal were treated with repeated injections of the corpuscles of another of different species, the serum of the former acquired a marked hæmolytic property towards the corpuscles of the latter, the property being demonstrated when the serum is added to the corpuscles. He also found that the hæmolytic property disappeared when the hæmolytic serum was heated at 55° C., but, as in the case of a bacteriolytic serum, was regained on the subsequent addition of some serum from a fresh (*i.e.* non-treated) animal. Ehrlich and Morgenroth analysed the phenomena in question, and showed that the specially developed and heat-resisting substance, "immune-body," entered into combination with the red corpuscles at a comparatively low temperature, namely, at 0° C.; whereas complement does not combine at this temperature. In this way a method is supplied by which the immune-body can be removed from a hæmolytic serum while the complement is left. They came to the conclusion that immune-body combines with the complement, though the combination is less firm and only occurs at a higher temperature—best about 37° C. They therefore considered that the immune-body acts as a sort of connecting-link between the red corpuscle and the complement, hence the term "amboceptor" which Ehrlich afterwards applied. It may be stated, however, that the direct union of complement and immune-body has not been conclusively demonstrated. Muir and Browning, for example, found that when a fresh serum is passed through a Berkefeld filter, complement is largely retained in the pores of the filter, whereas immune-body passes through practically unchanged; and that if a mixture of complement and immune-body be made and filtered at a temperature of 37° C., the amount of immune-body which passes through is not diminished, whereas it would be if it had united with the retained complement. Accordingly by this method there was obtained no evidence of the direct union of immune-body and complement. Bordet holds that the immune-body acts merely as a sensitising agent—hence the term *substance sensibilisatrice*—and allows the ferment-like complement to unite. It is quite evident from his

writings, however, that he does not mean, as is often assumed, that the immune-body causes some lesion in the corpuscle which allows the complement to act, but simply that it produces in the molecules (receptors) of the red corpuscles an avidity for complement. All that we can say definitely at present is that the combination of antigen+immune-body takes up complement in firm union, while neither does so alone. Even after the corpuscles are laked with water the receptors are not destroyed. Muir and Ferguson showed that they can still take up immune-body and, through its medium, complement, just as the intact corpuscles do. Ehrlich and Morgenroth showed that in some cases the red corpuscles can take up much more immune-body than is necessary for their lysis, and Muir found in one case studied, that each further dose of immune-body led to the fixation of more complement, so that as many as ten times the hæmolytic dose of complement might thus be used up. It is a matter of considerable importance that the union of immune-body and red corpuscles can be shown to be a reversible action. If, as was found by Morgenroth and Muir independently, corpuscles treated with several doses of immune-body and then repeatedly washed in salt solution, be mixed with untreated corpuscles and allowed to remain for an hour, then sufficient immune-body will pass from the former to the latter, so that all become lysed on the addition of sufficient complement. The combination of complement, on the other hand, is usually of very firm nature. It has been a disputed point whether there are several distinct complements in a normal serum with different relations to different immune-bodies, for which Ehrlich and his co-workers have brought forward a certain amount of evidence, or whether, as Bordet holds, there is a single complement, which may, however, show slight variations in behaviour towards different immune-bodies. There is at least no doubt that all the complement molecules in a serum are not the same. For example, Muir and Browning have shown that the treatment of a normal serum with a small amount of emulsion of a bacterium will remove the bactericidal action for another bacterium, whereas the amount of complement as tested by hæmolysis is practically unchanged. They accordingly consider that there is a moiety of complement, " bacteriophilic complement," which is specially concerned in bactericidal action. On the other hand, many of the arguments adduced by Ehrlich and his co-workers in favour of a multiplicity of complements are open to another interpretation; the truth probably lies between Ehrlich's and Bordet's views. Workers of the French school hold that comple-

ment does not exist in the free condition in the blood, but is liberated from the leucocytes when the blood is shed. This cannot be held as proved, and there are many facts against such a view. For instance, it was shown by Muir and M'Nee that the introduction of immune-body into the circulation of the corresponding animal leads to a lysis of red corpuscles by means of complement, that the lysis may go on gradually for a considerable period of time, and, further, that immune-body may become dissociated from red corpuscles and combine with others, the latter then undergoing lysis. These facts and many others speak strongly in favour of the view that complement exists in the free condition in the circulating blood. There is, however, evidence that the amount of free complement increases after the blood is shed and some time later gradually diminishes.

The hæmolytic action of a *normal* serum can be shown in some cases to be of the same nature as that of an immune-serum, that is, complement and the homologue of an immune-body can be distinguished. For example, guinea-pig's serum is hæmolytic to ox's corpuscles ; if a portion of serum be heated at 55° C., the complement will be destroyed ; if another portion be treated with ox's corpuscles at 0° C., the natural immune-body will be removed and only complement will be left. Neither portion is in itself hæmolytic, but this property becomes manifest again when the two portions are mixed. Hæmolytic sera are of great service in the study of the question of specificity. Each is specific in the sense already explained (p. 184), but the serum developed against the corpuscles of an animal may have some action on those of an allied species, that is, some receptors are common to the two species. This fact can be readily shown by the usual absorption tests, for example, in the case of an anti-ox serum tested on sheep's corpuscles. A close analogy holds to what has been established in the case of agglutinins. It is, further, of great interest to note that by the injection of red corpuscles into an animal its serum not only becomes hæmolytic, but in many cases when heated at 55° C. possesses also agglutinating and opsonic properties towards the red corpuscles used. These facts show how close an analogy obtains between antibacterial and hæmolytic sera, and how important a bearing hæmolytic studies have on the questions of immunity in general.

It has been shown by Forssman that a lysin for sheep's red corpuscles can be developed in the rabbit by the injection of emulsions of tissues of certain animals, *e.g.* guinea-pig, horse, etc. The antigens in these tissues are known as *heterogenetic* and the corresponding antibodies as *heterophile.* The nature of such antigens is still unknown, though there is evidence that they are lipoidal combinations. It has been found impossible, however, to obtain heterophile antibodies by the injection of separated lipoids alone, obtained by extracting the organs with alcohol. On the other hand, the antibodies, along with the lipoids in question, give reactions *in vitro*, *e.g.* fixation of complement and flocculation.

In addition to hæmolytic sera, antisera have been obtained by

the injection of leucocytes, spermatozoa, ciliated epithelium, liver cells, nervous tissue, etc. The laws governing the production and properties of these are identical, that is, each serum exhibits a specific property towards the body used in its production—*i.e.* dissolves leucocytes, immobilises spermatozoa, etc. The specificity is, however, not so marked as in the case of sera produced against red blood corpuscles ; thus a serum produced against tissue cells is often hæmolytic ; this is probably due to various cells of the body having the same receptors. Here again, when the antiserum produces no destructive effect on the corresponding cells, the presence of an immune-body may be demonstrated by the increased amount of complement which is taken up through its medium. It may also be mentioned that each antiserum usually exhibits toxic properties towards the animal whose cells have been used in the injections, *e g.*, a hæmolytic serum may produce a fatal result, with signs of extensive blood destruction, hæmoglobinuria, etc., *i.e.*, it is hæmotoxic for the particular animal ; a serum prepared by injection of liver cells has been found to produce on injection necrotic changes in the liver in the species of animal whose liver cells were used. These are mentioned as examples of a very large group of specific activities.

(*b*) *Opsonic Action.*—The presence of a substance in an immune-serum which makes the corresponding organism sensitive to phagocytosis was first demonstrated by Denys and Leclef in 1895, in the case of an anti-streptococcal serum. They also showed that the serum produced this effect by acting on the organism, not on the leucocytes. It is, however, chiefly to the researches of Wright and his co-workers that this subject has come into special prominence. Wright and Douglas in their first paper showed that the phagocytosis of staphylococci by leucocytes depended on a body in the normal serum which became fixed to the cocci and made them a prey to the phagocytes. To this they gave the name of " opsonin " (*vide* p. 128). There is no phagocytosis of cocci by leucocytes washed in salt solution ; normal serum heated to 55° C. is also without effect in inducing this phenomenon. They could not demonstrate any effect of the opsonin on the leucocytes. On the other hand, if bacteria be exposed to the fresh serum, and they be freed from the excess of serum and then exposed to leucocytes, also washed free from serum, they will be readily taken up by the cells. It has been shown that the opsonic action of the serum against an organism is increased by the process of immunisation, and the opsonic index represents the degree of immunity in one of its aspects, as already explained. In an immune-serum, however, an opsonin may still be present after the serum is heated at 55° C., as was shown by G. Dean and others ; and Muir and Martin showed that this thermostable immune-

opsonin (bacteriotropin of Neufeld) has all the specific characters of antibodies in general. On the other hand, they found that the thermolabile opsonin of a normal serum has quite different properties. For example, when a normal serum is tested on a particular bacterium, the opsonic effect on that bacterium may be removed by treating the serum with other bacteria; in other words, the thermolabile opsonin of normal serum does not possess the specific character of the opsonin developed in the process of immunisation. They have found, further, that various substances or combinations of substances which act as "complement absorbers" also remove the opsonic property from a normal serum, while they have no effect on an immune-opsonin.

That this thermolabile normal opsonin can act in a non-specific way is shown by the fact that particles of carmine and other substances become opsonised by the action of normal serum. It is, however, to be noted that in certain cases there have been found in a normal serum traces of substances which can be activated by thermolabile opsonin after the manner of immune-body and complement (as seen in the hæmolytic action of a normal serum, p. 200); to this extent the opsonic effect of a normal serum may have some degree of specificity. From this and other facts some observers have attempted to explain the whole of opsonic action according to the scheme of *immune-body + complement* as seen in hæmolysis. This, however, is not justifiable, since normal thermolabile opsonin can, as we have seen, act by itself, as can also the specific immune-opsonin after normal opsonin has been destroyed by heating. The subject is one of considerable complexity, but it may be said that the most important variations in the opsonic content observed in infections depend on the specific immune-opsonins, though the presence of immune-body may play a part in raising the index, by leading to the union of more normal-complement-opsonin.

(*c*) *Agglutination.*—Charrin and Roger in 1889 observed that when the bacillus pyocyaneus was grown in the serum of an animal immunised against this organism, the growth formed a deposit at the foot of the vessel; whereas a growth in normal serum produced a uniform turbidity. Gruber and Durham, in investigating Pfeiffer's reaction, found that when a small quantity of an antiserum is added to an emulsion of the corresponding bacterium, the organisms become agglutinated into clumps, this phenomenon depending upon the presence of bodies in the serum called *agglutinins*.

It had already been found that the serum of convalescents from typhoid fever could protect animals to a certain extent against typhoid infection, and, in view of the facts experimentally established, it appeared a natural proceeding to inquire

whether such serum possessed an agglutinative action and at what stage of the disease it appeared. The result, obtained independently by Grünbaum and Widal, but first published by the latter, was to show that the serum possessed this specific action shortly after infection had taken place; in other words, the development of this variety of antibody can be demonstrated at an early stage of the disease. Agglutination may be said to be observed generally in bacterial infections, though the degree of the phenomenon and the facility with which it can be noted vary greatly in different cases. Details will be found in the chapters dealing with individual diseases, etc. Furthermore, the phenomenon is not peculiar to bacteria; it is seen, for example, when an animal is injected with the red corpuscles of another species, *hæmagglutinins* appearing in the serum, which have a corresponding specificity, as was first shown by Bordet.

The physical changes on which agglutination depends cannot as yet be said to be fully understood. It was shown by Nicolle and by Kruse that if an old bacterial culture be filtered through porcelain, the addition of some of the corresponding antiserum produces a sort of granular precipitate in it; and that when minute inorganic particles are added to the mixture, they become aggregated into clumps, as in the agglutination of bacteria. The phenomenon would thus appear to be the result of the interaction of the agglutinin and some substance in the bacterial cell which is known as the agglutinable substance or as the agglutinogen, the resulting effect being allied to precipitation. Another factor necessary for the phenomenon of agglutination is suitable salt content. Bordet showed that when the bacterial emulsion and the agglutinin are made salt-free by dialysis, agglutination does not take place on their being mixed, though it does so on the addition of an electrolyte such as sodium chloride; and, further, that if the clumps of agglutinated bacteria are freed from salt by washing in distilled water they become resolved, and that on the addition of some sodium chloride they are formed again. Joos also has brought forward striking confirmatory evidence as to the necessity for the presence of salts.

In the phenomenon of agglutination we have thus to distinguish two factors, namely (*a*) the combination of agglutinin and agglutinable substance (agglutinogen), and (*b*) the actual clumping of the bacteria; and it is to be noted that whether or not the latter event follows, depends on the physical condition of each of the two substances concerned, as well as on the presence of electrolytes. For example, in some cases when the

bacteria are heated at a temperature of 65° C. for some time, they may lose the faculty of being agglutinated while they may still retain the property of combining with or binding agglutinin. Dreyer and Jex-Blake have observed the remarkable fact that in certain instances on being heated to a still higher temperature they may once more become agglutinable. Another point of practical importance is that bacteria when freshly grown from the tissues are very often less agglutinable than they afterwards become when subcultured for some time. The nature of agglutination will be discussed further after precipitation has been considered (p. 207).

As stated above, the agglutinins are regarded as possessing a combining group and an active or agglutinating group. The constitution would thus be analogous to that of a toxin, and in conformity with this view, Eisenberg and Volk consider that the agglutinating group may be destroyed while the combining group remains, the result being an *agglutinoid*. The evidence for this lies in the fact that when an agglutinating serum is heated to a certain temperature, not only does it lose its agglutinating action, but when the bacteria are treated with such a serum, their agglutination by active serum is interfered with, a sort of plugging-up of the combining molecules having apparently taken place. Again, with agglutinating sera partially inactivated by heat or other means, what are known as "zone phenomena" occur; that is, when agglutination occurs with a given dilution of such a serum, a lower dilution may fail to agglutinate, and this they suppose to be due to the interference of the union of agglutinin by agglutinoid in the greater concentration of serum. On the other hand, there are facts which cannot be brought into harmony with this view. For example, Dreyer and Jex-Blake showed that the inhibition zone may be slight when there has been much destruction of agglutinin, and, on the other hand, may be well marked when no weakening of the agglutinating power has resulted from the heating. The physical changes underlying such phenomena are still very obscure, but we may say at present that the existence of agglutinoids has not yet been proved. Such zone phenomena are frequently observed in the interaction of colloid substances (pp. 197, 208).

Like immune-bodies, agglutinins are not destroyed at 55° C. (a temperature sufficient to annul bactericidal action), but different agglutinins show variations in this respect, some being affected by a temperature little above that named. The resistance to heat also varies when the serum is diluted with salt solution, and it has been shown that conditions which interfere with the coagulation of the proteins increase their resistance. Like antitoxins, agglutinins seem to be chiefly contained in the globulin fraction. Discussion has taken place as to the relation of agglutinins to immune-bodies and as to how far agglutination

is an indication of immunity. It may be said that the two properties, agglutination and fixation of complement, do not always run parallel; in fact, many striking examples to the contrary might be given. An antibacterial serum may exhibit marked agglutinative properties, but with the complement-fixation function poorly developed; and the converse may be noted. But, as already stated, we are dealing with properties rather than with substances, and in connection with any effect both antigen and antibody are concerned. Non-agglutination may be due to a peculiarity in the bacteria, as well as to an absence of agglutinin. Agglutinins become fixed in definite proportion by the receptors of the bacteria—that is, the agglutinin becomes used up in the process of agglutination; and it has been shown that bacteria may take up many times the amount necessary to their agglutination—a corresponding fact to what has been established with regard to immune-bodies of hæmolytic sera. The agglutinins are specific in the sense which has been explained above (p. 184). It can be shown by the method of absorption that in an agglutinating serum there may be several agglutinins with different combining groups, some of which may be taken up by organisms allied to that which has given rise to the antiserum (p. 185).

Besides those stated above, other phenomena have been observed in the interaction of antisera and the corresponding bacteria. For example, it has been shown that when certain bacteria—*e.g.* the typhoid bacillus, B. coli, and B. proteus—are grown in bouillon containing a small proportion of the homologous serum, their morphological characters may be altered, growth taking place in the form of threads or chains which are not observed in ordinary conditions. In other instances a serum may inhibit some of the vital functions of the corresponding bacterium.

Precipitins.—Shortly after the discovery of agglutinins, Kraus showed in the case of the organisms of typhoid, cholera, and plague, that the antiserum not only caused agglutination, but when added to a filtrate of a culture of the corresponding bacterium, produced a cloudiness and afterwards a precipitate. To the substance in the immune-serum which brought about this effect he gave the name of *precipitin*. Subsequent study has shown that this phenomenon is closely related to agglutination; in fact, several authorities consider that they represent the same reaction under different conditions—that is, that the substances which, when present in the bacterial bodies, give rise to agglutination, on the addition of the antiserum, produce a precipitate when free in a fluid. To test the reaction it is

accordingly necessary to have as far as possible the substance of the bacteria in solution, and for this purpose there have been introduced various methods, of which the two following may be given :

(*a*) It is well known that in an old bouillon culture the bacteria undergo disintegration and their constituents go into solution. Accordingly, if such a culture which has been kept in the incubator for several weeks be filtered through a porcelain filter, the filtrate will contain the reacting substance or precipitinogen.

(*b*) The growth from a recent agar culture is scraped off and suspended in normal salt solution, the mixture is made feebly alkaline with soda solution and boiled for a few minutes. The mixture is then neutralised, when a precipitate forms, and is filtered through filter paper ; the filtrate contains the precipitinogen.

The precipitin test is carried out by placing in a number of small test-tubes a given amount of the bacterial filtrate along with varying quantities of the homologous antiserum. (The latter may be obtained in the usual way by the repeated injection of dead cultures or of bacterial filtrate.) As the precipitate forms slowly the tubes should be placed in the incubator for twenty-four hours, 0·5 per cent. carbolic acid being added to prevent the growth of bacteria. This precipitin reaction has now been observed in a great many bacterial diseases when the patient's serum is added to the corresponding bacterial filtrate, and has even been applied by some observers as a means of diagnosis. It is, however, less delicate and more restricted in its application than the agglutination methods.

Serum Precipitins.—This subject does not strictly belong to bacteriology, but the general phenomena are so closely allied to those just described that some reference may be made to it. When the serum of an animal is injected in repeated doses into another animal of different species, after the type of an immunisation, there appears in the serum of the animal treated a substance called precipitin, which causes a cloudiness or precipitate when added to the serum (precipitinogen) used. (In the case of rabbits, doses of 3 to 4 c.c. of the serum may be injected intraperitoneally at intervals of four to five days, a precipitin usually appearing at the end of about three weeks.) The reaction, which is a very delicate one, is conveniently observed by adding a given amount of the antiserum, say 0·05 c.c., to varying amounts of the homologous serum 0·1, 0·01, etc., c.c., in a series of small test-tubes, the volume being then made up with salt solution to 1 c.c. In this way a definite reaction may be observed with 0·0001 c.c. of the homologous serum or even less. An even more delicate reaction is obtained when the solution of antigen is placed with a pipette on the surface of the solution of precipitin, a layer of cloudiness then forming at their junction—the so-called " ring " test. Here again zone phenomena, as in the

case of agglutination, are met with. If the antiserum be heated to a temperature of 75° C. for some time it acquires inhibitory properties, so that when added to a mixture of serum and antiserum which would otherwise give a precipitate, this no longer occurs. Some observers consider that this is due to the presence of "precipitoid" in the heated antiserum; but the observations of Welsh and Chapman show that this view is not in accordance with the facts, and indicate that the inhibition is related to a specific solvent action which the heated antiserum has on the precipitate. They have also shown that the main mass of the precipitate is furnished by the antiserum (precipitin), and not as was usually supposed by the protein of the homologous serum thrown down by the precipitin; this result is of high importance in connection with the action of anti-substances in general. The precipitin reaction is specific in the sense explained above. It is always most marked towards the serum of the species used in the immunisation; but while this is so, there may also be a slight reaction towards animals of allied species. An anti-human serum, for example, gives the maximum reaction with human serum, but also a slight reaction with the serum of monkeys, especially of anthropoid apes; it, however, gives no reaction with the serum of other animals. The precipitin test has thus come to be employed as a means of differentiating human from other bloods. Another interesting phenomenon is what is known as the "fixation of complement," which is produced by the combination of the two substances in the serum and antiserum respectively. If mixtures be made according to the above method, and then a small quantity of complement, say fresh guinea-pig serum, be added, it will be found that the complement becomes absorbed, as may be shown by subsequently adding a test amount of sensitised red blood corpuscles. This fixation phenomenon is even a more delicate reaction than the precipitin test, it being often possible to demonstrate by its use from a tenth to a hundredth of the smallest amount of serum which will give a perceptible precipitate; it also is specific within the same limits.

Nature of Agglutination and Precipitation.—All are agreed that agglutination and precipitation are closely allied phenomena and present certain similarities to well-known reactions exhibited by colloids. In both there is a reduction in the dispersion of colloids, these being in agglutination in the form of particles in suspension, and in precipitation as colloidal solutions. It is recognised that the chief force which keeps particles or molecules apart in a fluid is the like electric charge which they bear, while surface tension is the force which tends to draw them together. The combination with molecules of opposite charge will thus tend to produce aggregation; and it is known that when the colloid added is insufficient to cause precipitation or flocculation, this may occur on the addition of an electrolyte. For example, a solution of gelatin and gum mastic may be prepared in such proportions that precipitation occurs when a weak solution of

electrolyte such as sodium chloride is added. In this there is a close analogy to what has been described above in the case of agglutination. Another striking resemblance to colloidal interaction is seen in the *zone phenomena*. For example, when the amount of precipitin is kept constant in a series of tubes and gradually increasing amounts of precipitinogen (antigen) are added, the resulting precipitate increases up to a point, but beyond that point increased amounts of antigen cause diminution of the precipitate, and ultimately its disappearance. A similar zone phenomenon is seen when, for instance, increasing amounts of a colloidal solution of iron hydroxide are added to a mastic emulsion. Such a phenomenon has been explained as being due to alteration of the electric charge of the particles of the one colloid by the added particles of the other, till the charge is brought to zero; there then occurs the maximum precipitate. Further addition leads to reversal of the charge, and thus once more to dispersion of the molecules. Other somewhat similar explanations of the phenomena of agglutination and precipitation have been put forward, but no one view has yet received general acceptance. Even if it is the case, as seems certain, that the physical phenomena correspond to the behaviour of other colloids, it must be borne in mind that the essential factor is the *specific* combination of antigen and antibody—of agglutinogen and agglutinin, of precipitinogen and precipitin; and to this there is no analogy in colloidal reactions so far as we know. The specific combination of agglutinin with bacteria occurs in water free from electrolytes; though the result of this combination is not manifest until an electrolyte is added. Some writers consider that chemical changes are superadded to colloidal reactions, but it seems to us that the first and all-important change is the combination of the antigen with the anti-substance, and the specific character here seems to depend on chemical peculiarities of the molecule. Whether some recognisable physical change follows thereafter depends on various circumstances, especially on the proportions in which the two substances are present and on the presence or absence of electrolytes.

The Source and Nature of Antibodies.—The earlier work on these questions was concerned mainly with antitoxins, and various theories were put forward. One of the first views to be advanced was that antitoxin molecules represented toxin molecules which had been in some way modified by the cells of the body; but it was soon established that this view could not be maintained. It was found that the amount of antitoxins

produced by an animal may be many times greater than the equivalent of toxin injected; and, further, that when an animal is bled the total amount of antitoxin in the blood may some time afterwards be greater than it was immediately after the bleeding, even although no additional toxin is introduced. The latter circumstance shows that antitoxin is *formed* by the cells of the body. This being so, it remains to be determined whether it is a normal constituent of the cells which is formed in increased quantity or whether it is a new product. We have, however, direct evidence of the presence of antitoxin under normal conditions—the presence of such being shown by its uniting with toxin and rendering it inert. Normal horse serum, to mention an example, may have a varying amount of antitoxic action to the diphtheria poison, whilst in the case of other antibodies—such as agglutinins, bacteriolysins, hæmolysins, etc.—whose production is governed by the same laws, numerous examples might be given. The presence of representatives of a great multiplicity of antibodies in normal sera is a circumstance of great significance, as these are undoubtedly the products of cellular activity, and in all probability molecules of corresponding nature occur as constituents of cells; an increased formation and setting free of these may, therefore, explain the production of antibodies in active immunity. But have all the antibodies normal representatives? If not, then the molecules of antigen must by a sort of impress alter the configuration of molecules in the cells so that they function as antibodies. Regarding such an occurrence, however, we know nothing. As to the manner in which the antigens influence the cells, we have evidence that in the living body bacterial toxins enter into combination with, or, as it is often expressed, are fixed by the tissues—presumably by means of certain combining affinities. This has been shown by the experiments of Dönitz and of Heymans with tetanus toxin. We have in such cases no evidence as to where the toxin is fixed beyond that supplied by the occurrence of symptoms. We may note, however, that it is not a serious objection, that in certain animals other tissues than that of the central nervous system can combine with tetanus toxin—this might take place with or without resulting symptoms. It is, moreover, evident that the molecules in the cells which unite with toxin may, when set free, act as antitoxin by neutralising the toxin and thus preventing its combination with the cells. This will be referred to below in connection with Ehrlich's theory (p. 214).

Another line of research which has been followed is to bring emulsions of various organs into contact with a given toxin and observe whether any of the toxicity is removed. This was first carried out by Wassermann and Takaki, who investigated the action of emulsions of the central nervous system of the susceptible guinea-pig on tetanus toxin. They found in this way that the nervous system contained bodies which had a neutralising effect on the toxin. For example, it was shown that 1 c.c. of emulsion of brain and spinal cord was capable of protecting a mouse against ten times the fatal dose of toxin. These observations have been confirmed, though their significance has been variously interpreted; and in view of the ascertained facts with regard to processes of physical absorption, it is quite possible that this neutralisation of toxin does not represent a specific union as in the case of antitoxic action.

With regard to the sites of origin of other antibodies our information is still very deficient. Pfeiffer and Marx brought forward evidence in the case of typhoid, and Wassermann in the case of cholera, that the immune-bodies are chiefly formed in the spleen, lymphatic glands, and bone-marrow. According to certain workers of the French school, the chief source of antibodies acting on cells such as red blood corpuscles is the large mononuclear leucocytes, whilst those acting on bacteria are chiefly derived from the polymorpho-nuclear leucocytes (p. 159). Another view is that immune-bodies are chiefly formed by the large mononuclear leucocytes, whilst complements are products of the polymorphs. That these cells are concerned in the production of antagonistic and protective substances is almost certain, though another possible source of wide extent, namely, the endothelium of the vascular system, has been largely overlooked. In recent times the reticulo-endothelial system has been held to be an important source of antibodies, and various observations have been adduced in support of such a view; as yet, however, definite statements cannot be made on this point. It is to be noted, however, that antibodies have never been extracted from leucocytes or other cells, and a similar statement holds with regard to complement.

Of the *chemical nature of antibodies* we know little. From their experiments, C. J. Martin and Cherry deduced that while toxins are probably of the nature of albumoses, the antitoxins probably have a molecule of greater size, and may be allied to the globulins. Hiss and Atkinson also came to the conclusion that antitoxin belongs to the globulins. They found that the precipitate with magnesium sulphate from anti-diphtheria serum contained practically all the antitoxin, and that any substance obtained which had an antitoxic value gave all the reactions

of a globulin ; and this result has been confirmed by others. They found also that the percentage amount of globulin precipitated from the serum of the horse increased after it was treated in the usual way for the production of antitoxin. Ledingham observed an increase of globulin during the process of immunisation of a horse which yielded a high-grade antitoxic serum, and he ascertained that while this increase was more on the part of the euglobulin than of the pseudoglobulin fraction, most of the antitoxin was contained in the latter ; hence a method of concentrating antitoxin is supplied.

Similar results have been obtained with regard to the other classes of antibodies. They are mostly found along with the globulins, though the fraction with which they are chiefly associated varies in different cases. From the evidence established, it appears likely that anti-substances are really globulins, but this cannot be definitely stated, as they have not yet been obtained in pure form. It may be mentioned in this connection that Huntoon and his co-workers have separated antibodies from their combination with pneumococci, and have found that they do not correspond in their reactions with serum proteins ; for example, they are not affected by trypsin and are not precipitated like globulins by saline solutions.

Antitoxin, when present in the serum, leaves the body in various secretions, and in these it has been found, though in much less concentration than in the blood. It is present in the milk, and a certain degree of immunity can be conferred on animals by feeding them with such milk, as has been shown by Ehrlich, Klemperer, and others. Klemperer found traces of antitoxin in the yolk of eggs of hens whose serum contained antitoxin. Bulloch also found in the case of hæmolytic sera that the antibody is transmitted from the mother to the offspring.

Non-Specific Formation of Antibodies.—As stated above, it is clear that antibodies are formed by the cells of the body, and it has now been shown that their formation can be influenced by factors other than the injection of antigens. For example, it has been found that if the blood of an animal containing antibodies is removed and replaced by the blood of a normal animal, the antibodies are rapidly restored. And further, repeated small bleedings may raise the content of the blood in antibodies above its former level. Acting on the supposition that antitoxin formation is allied to a process of secretion, Salamonsen and Madsen tested the effect of pilocarpine and found that it had the property of producing a marked rise in the amount of antitoxin present in the blood of an animal. Walbum and others

have also tested the effects of various metallic salts and have found that many have a similar property of stimulating the formation of antibodies. These were tested on immunised animals after the antibody content had fallen to a steady level. Salts of different metals vary much in their action, but they found that amongst the metals of the iron group manganese chloride has the greatest effect, this salt sometimes rapidly raising the amount of antibodies almost to the previous maximum. The most efficient salt of all, however, was beryllium chloride. Mackie has shown that the normal antibody of the rabbit for sheep's corpuscles is increased by extensive bleedings and also by the administration of metallic salts (manganous chloride and beryllium chloride), colloidal manganese, salvarsan, etc. The explanation of such non-specific stimulation is quite obscure.

Therapeutic Use of Antisera.—As will have been gathered, the chief human diseases treated by antitoxic sera are diphtheria, tetanus, botulismus, snake-bite, and scorpion sting; and by antibacterial sera, streptococcus infection, cerebro-spinal fever, pneumonia, dysentery, and plague. The methods of application in bacterial infections and the general results have been dealt with in treating of individual diseases. In snake-bite the use of antivenenes is limited, for Lamb showed that, if a cobra with full glands bites a man, many times the minimal lethal dose are probably injected. Grave symptoms thus come on so rapidly that usually no opportunity is offered for remedial treatment by the antisera. Moreover, as a definite specificity exists between the poison of a particular snake and its antivenene, unless the appropriate serum is available, little effect will be produced. In cases of slight bite, however, benefit may accrue from the use of the antiserum.

As has been shown above, antibacterial sera require for their bactericidal action a sufficiency of complement, and as this becomes lost when a serum is kept, the unsatisfactory results with this class of sera may be due to a deficiency of complement. Or it may be, as Ehrlich suggested, that the complement naturally existing in human serum does not suit the immune-body in the antiserum—that is, is not taken up through the medium of the latter and brought into combination with the bacterium. And there is the further possibility that even though the complement should be taken up, the zymotoxic group of the latter is not sufficiently active towards the bacterium to effect its death. In both cases it will appear that an extracellular bactericidal action cannot be produced by the

particular immune-body in association with the complement of the animal in question. There is no doubt that this question of complements is one of high importance, and that both combining affinity and toxic action of complements must be considered in each case.

In such diseases as cerebro-spinal fever and pneumonia the opsonic mechanism of the infected individual may play a part in successful resistance. The favourable effects following treatment with antisera may thus, in some cases, depend on an augmentation of the opsonic powers of the body.

Theories as to Acquired Immunity

The advances made within recent years in our knowledge regarding artificial immunity, and the methods by which it may be produced, have demonstrated the insufficiency of various theories which had been propounded. Only a short reference need be made to these.

The *theory of exhaustion*, with which Pasteur's name is associated, supposed that in the body of the living animal there are substances necessary for the existence of a particular organism, which become used up during the sojourn of that organism in the tissues; this pabulum being exhausted, the organisms die out. Such a supposition is, of course, quite disproved by the facts of passive immunity. According to the *theory of retention*, the bacteria within the body were considered to produce substances which are inimical to their growth, so that they die out, just as they do in a test-tube culture before the medium is really exhausted. Such a theory only survives now in the view that antitoxins are modified toxins, the evidence against which has already been discussed (p. 208). There then came the *humoral theory* and the *theory of phagocytosis*, but neither of these is tenable in its pure form, and the distinction between them need not be maintained. For, on the one hand, any substance with specific property in the serum must be the product of cellular activity, and, on the other hand, the facts with regard to passive immunity go far beyond the ingestive and digestive properties of phagocytes, though these cells may be in part the source of important bodies in the serum.

At the present time interest centres around two theories, namely, Ehrlich's side-chain theory and Metchnikoff's phagocytic theory as further developed. These will now be discussed, and it may be noted that the ground covered by each is not coextensive. For the former deals chiefly with the production of anti-substances and their biological significance, the latter deals with the defensive properties of cells, either directly by their phagocytic activity or indirectly by substances produced

by them after the manner of digestive ferments. It will be seen, however, that each has a normal process as its basis, namely, that of nutrition.

Ehrlich's Side-Chain Theory.—This may be said to be an application of his views regarding the nourishment of cells. A molecule of protoplasm (in the general sense) may be regarded as composed of a central atom group or functional centre with a large number of side-chains, *i.e.* atom groups with combining affinity for food-stuffs. It is by means of these latter that the living molecule is increased in the process of nutrition, and hence the name *receptors* given by Ehrlich is on the whole preferable. In considering the application of this idea to the facts of acquired immunity, it must be kept in view that all the substances to which antibodies have been obtained are, like proteins, of unknown but undoubtedly of complex chemical constitution, and that in apparently every case the antibody enters into combination with its corresponding antigen. The dual constitution of toxins and kindred substances, as already described (p. 172), is also of importance in this connection. Now, to take the case of toxins, when these are introduced into the system they are fixed, like food-stuffs, by their haptophore groups to the receptors of the cell protoplasm, but are unsuitable for assimilation. If they are in sufficiently large amount, the toxophore part of the toxin molecule produces that disturbance of the protoplasm which is shown by symptoms of poisoning. If, however, they are in smaller dose, as in the early stages of immunisation, fixation to the protoplasm occurs in the same way; and as the combination of receptors with toxin is supposed to be of firm nature, the receptors are lost for the purposes of the cell, and the combination R.-T. (receptor+toxin) is shed off into the blood. The receptors thus lost become replaced by new ones, and when additional toxin molecules are introduced, these new receptors are used up in the same manner as before. As a result of this repeated loss, the regeneration of the receptors becomes an over-regeneration, and the receptors formed in excess appear in the free condition in the blood stream and then constitute antitoxin molecules. There are thus three factors in the process, namely (1) fixation of toxin, (2) over-production of receptors, (3) setting free of receptors produced in excess. Accordingly, these receptors which, when forming part of the cell protoplasm, anchor the toxin to the cell and thus are essential to the occurrence of toxic phenomena, in the free condition unite with the toxin, and thus prevent the toxin from combining with the cells and exerting a pathogenic action. Ehrlich did not state what

cells are specially concerned in the production of antibodies, but from what has been stated it is manifest that any cell which fixes toxin is potentially a source of antitoxin. Cells to whose disturbance, resulting from the fixation of toxin, characteristic symptoms of poisoning are due, ought thus to be sources of antitoxin, *e.g.* cells of the nervous system in the case of tetanus, though the cells not seriously affected by toxin fixation may act in the same way. The experimental investigation of the source of antitoxins has, however, yielded little result, and no definite statement can be made on the subject.

It will be seen that Ehrlich's theory accords well with many of the known facts of active and passive immunity, and up to a point it affords an explanation of the multiplicity of antibodies. For, if we take the case of antitoxins, we see that this depends upon the combining affinity of the toxin for certain of the cells of the body, and this again is referred back to the complicated constitution of living protoplasm. It is to be noted, however, that it does not explain active immunity apart from the presence of antibodies in the serum. For example, an animal may be able to withstand a much larger amount of toxin than could be neutralised by the total amount of antitoxin in its serum, or may possess immunity when antibodies have disappeared from the blood. This might theoretically be explained by supposing a special looseness of the cell receptors so that the toxin-receptor combination became readily cast off. The question, however, arises whether there may not be really an increased resistance of the cells to the toxic action. An observation made by Meyer and Ransom (*vide* p. 535) is also difficult of explanation, according to the view that antitoxin is formed by the cells with which the toxin combines and on which it acts. They found that in an animal actively immunised against tetanus and with antitoxin beginning to appear in its blood, the injection of a single M.L.D. of tetanus toxin into a peripheral nerve brought about tetanus with a fatal result. On the other hand, the injection of antitoxin into the sciatic nerve above the point of injection of toxin prevented the latter from reaching the cells of the cord. One can scarcely imagine an explanation of these facts if antitoxin molecules were in process of being shed off by the cells of the nervous system. Further, when the serum of an animal contains a large amount of antitoxin, how does the additional toxin injected reach the cells in order to influence them as we know it does? This also is difficult to understand, unless the toxin has a greater affinity for the receptors in the cells than for the free receptors (antitoxin) in

the serum. Further investigation alone will settle these and various other disputed points, and may remove many of the apparent objections. At present we may say, however, that Ehrlich's theory is the only one which even attempts to explain the cardinal facts of this aspect of immunity.

The Theory of Phagocytosis.—This theory, brought forward by Metchnikoff to explain the facts of natural and acquired immunity, has been of enormous influence in stimulating research on the subject. Looking at the subject from the standpoint of the comparative anatomist, he saw that it was a very general property possessed by certain cells throughout the animal kingdom, that they should take up foreign bodies into their interior and in many cases digest and destroy them. On extending his observations to what occurred in disease, he came to the conclusion that the successful resistance of an animal against bacteria depended on the activity of certain cells called phagocytes. In the human subject he distinguished two chief varieties, namely (*a*) the microphages, which are the "polymorpho-nuclear" finely granular leucocytes of the blood, and (*b*) the macrophages, which include the larger hyaline leucocytes, endothelial cells, connective tissue corpuscles, and, in short, any of the larger cells which have the power of ingesting bacteria. Insusceptibility to a given disease is indicated by a rapid activity on the part of the phagocytes, different varieties being concerned in different cases—an activity which may quickly destroy the bacteria and prevent even local damage. Phagocytosis was regarded by Metchnikoff as the essence of inflammation. If the organisms are introduced into the tissues of a moderately susceptible animal, there occurs an inflammatory reaction with local leucocytosis, which results in the intracellular destruction of the invading organisms. He also showed that the bacteria may be in a living and active state when they are ingested by leucocytes. On the other hand, he found that in a susceptible animal phagocytosis did not occur or was only imperfect; and further, that when a naturally susceptible animal was immunised, the process was accompanied by the appearance of an active phagocytosis. It is known that amœbæ and allied organisms have digestive properties which are specially active towards bacteria, and from what can be directly observed, as well as indirectly inferred, there can be no doubt that such a faculty is also possessed by the phagocytes of the body. Thus bacteria within these cells are in a position favourable to their destruction, and do in many instances become destroyed. In fact, observations on phagocytosis *in vitro*

show that such destruction may in the case of some organisms occur so rapidly that the actual number observable in the leucocytes is no indication of the activity of the process. In other instances, *e.g.* in gonorrhœa, the ingested organisms would appear to survive a considerable time without undergoing change. Undoubtedly phagocytosis is of the highest importance in active immunity, as by its means organisms which would not undergo an extracellular death may be killed off. In the process of immunisation of a susceptible animal we see a negative or neutral chemotaxis becoming replaced by positive chemotaxis. This was explained by Metchnikoff as due to an education or stimulation of the phagocytes. The work on opsonins shows, however, that this is not the case, as leucocytes from an immunised animal are, as a rule, not more active in this direction than those of a normal animal, the all-important factor being the development of an opsonin in the immune animal. Thus this phase of immunity comes to be merely an aspect of the action of antibodies in general.

The digestive ferments of phagocytes or *cytases* are, according to Metchnikoff, retained within the cells under normal conditions, but are set free when these cells are injured—for example, when the blood is shed. They then become free in the serum by the breaking up of the cells—the process known as phagolysis—and they then constitute the alexins, or complements of Ehrlich. Of these, as has already been said, Metchnikoff believed there are probably two kinds—one called *macrocytase*, contained in the macrophages, which is specially active towards the formed elements of the animal body, protozoa, etc. ; and the other, *microcytase*, contained within the polymorpho-nuclear leucocytes, which has a special digestive action on bacteria. It is the microcytase which gives blood serum its bactericidal properties. It appears to us, however, that Metchnikoff went too far in distinguishing the activities of the two classes of cells so sharply as he did.

With regard to the properties of antibacterial sera in relation to phagocytosis, Metchnikoff gave the following explanation. He admitted that the immune-body is fixed by the bacteria (or red corpuscles, as the case may be), though he did not state that a chemical combination takes place ; hence he called it a fixative (*fixateur*). The immune-bodies are to be regarded as auxiliary ferments (*ferments adjuvants*) which aid the action of the alexin. Unlike the latter, however, they are formed in excess during immunisation and set free in the serum. He compared their action to that of enterokinase, a ferment which is produced in the intestine and which aids the action of trypsin. Thus, when

the bacteria have fixed the immune-body, their digestion is facilitated either within the phagocytes, or outside of them when the alexin has been set free by phagolysis. He, however, maintained that extracellular digestion or lysogenesis does not take place without the occurrence of phagolysis. The source of immune-bodies is, in all probability, also the leucocytes, as these substances are specially abundant in organs rich in such cells—spleen, lymphatic glands, etc.; here again the mononuclear leucocytes are probably the source of the immune-bodies concerned in hæmolysis, the polymorpho-nuclear leucocytes the source of those concerned in bacteriolysis. Although the immune-bodies exist in the blood plasma, he held that this is not always the case; sometimes they are contained in the cells, and this probably occurs when there is a high degree of active immunity against bacteria without a serum having an antibacterial action, the powers of intracellular digestion being in such cases increased. In this way the facts of immunity can be explained so far as these concern the destruction of bacteria.

Metchnikoff's work has less direct bearing on the production of antitoxins. He admitted the fixation of the toxin by the antitoxin to form a neutral compound, and he apparently considered that leucocytes may also be concerned in the production of antitoxins. Apart, however, from antitoxin formation, he considered the acquired resistance of the cells themselves of high importance in antitoxic immunity.

When we consider Metchnikoff's theory as thus extended to cover recently established facts, it must be admitted that it affords a rational explanation of a considerable part of the subject, though the fact that the chemotactic phenomena during immunisation depend mainly on opsonins (antibodies), detracts from the importance which he attached to the phagocytes. It, however, does not afford explanation of the multiplicity and specificity of antitoxins; on the other hand, it is more concerned with the cells of the body as destroyers or digesters of bacteria. As regards the subject of antibacterial sera, the results of these two workers may be said to be in harmony in some of the fundamental conceptions. And it is of interest to note that Metchnikoff, starting with the phenomena of intracellular digestion, arrived at the idea of specific ferments given off by phagocytes; whilst Ehrlich, from his first investigations on the constitution of toxins, reached an explanation of antitoxins and immune-bodies also with a theory of cell-nutrition as its basis.

Natural Immunity

We have placed the consideration of this subject after that of acquired immunity, as the latter supplies facts which indicate in what direction an explanation of the former may be looked for. There may be said to be two main facts with regard to natural immunity. The first is, that there is a large number of bacteria —the so-called non-pathogenic organisms—which are practically incapable, unless perhaps in very large doses, of producing pathogenic effects in any animal; when these are introduced into the body they rapidly die out. This fact, accordingly, shows that the animal tissues generally have a remarkable power of destroying living bacteria. The second fact is, that there are other bacteria which are very virulent to some species of animals, whilst they are almost harmless to other species; the anthrax bacillus may be taken as an example. Now it is manifest that natural immunity against such an organism might be due to a special power possessed by an animal of destroying the organisms when introduced into its tissues. It might also possibly be due to an insusceptibility to, or power of neutralising, the toxins of the organism; for the study of the various diseases shows that the toxins (in the widest sense) are the weapons by which morbid changes are produced, and that toxin-formation is a property common to all pathogenic bacteria. As a matter of fact, however, natural immunity is, in most cases, one against *infection*, *i.e.* consists in a power possessed by the animal body of destroying the living bacteria when introduced into its tissues: such a power may exist though the animal is still susceptible to the separated toxins. We shall now look at these two factors separately.

1. *Variations in Natural Bactericidal Powers.*—The fundamental fact here is that a given bacterium may be rapidly destroyed in one animal, whereas in another it may rapidly multiply and produce morbid effects. The special powers of destroying organisms in natural immunity have been ascribed to (*a*) phagocytosis, and (*b*) the action of the serum.

(*a*) The chief factors with regard to phagocytosis have been given above. The bacteria in a naturally immune animal, for example, the anthrax bacillus in the tissues of the white rat, are undoubtedly taken up in large numbers and destroyed by the phagocytes, whereas in a susceptible animal this only occurs to a small extent; and Metchnikoff showed that they are taken up in a living condition, and are still virulent when tested in a

susceptible animal. Variations in phagocytic activity are found to correspond more or less closely with the degree of immunity present, but are probably in themselves capable of explanation. The fundamental observations of Wright and Douglas (p. 201) show that an essential in phagocytosis is the labile opsonin of normal serum, which has combining affinities for a great many organisms, as already stated. In other cases, more specific substances may be concerned. But the all-important fact is that whether phagocytosis occurs or not appears to depend upon certain bodies in the serum. As yet, we cannot say whether the phagocytosis in a given serum, observed according to the opsonic technique, always runs parallel with phagocytosis in the tissues of the animal from which the serum has been taken. But whether or not phagocytosis *in vivo* corresponds with that *in vitro*, it is probably to be explained in the same way ; that is, it probably depends upon the content of the serum. The composition of the latter, no doubt, is the result of cellular activity, and in this the leucocytes themselves are in all probability concerned, but the movements and phagocytic activity of these cells seem to be chiefly, if not entirely, controlled by their environments. Ingestion is, however, only the first stage in the process : intracellular destruction is the second, and is of equal importance. What may be called intracellular bactericidal action probably varies in the case of leucocytes of different animals, but regarding this our knowledge is deficient, and, further, bacteria sometimes survive the cells which have ingested them, the latter undergoing necrosis and disintegration. In other instances, the organisms do not appear to suffer from their intracellular position ; an example of this is afforded in the case of gonococci.

(*b*) When it had been shown that normal serum possessed bactericidal powers against different organisms, the question naturally arose as to whether this bactericidal power varied in different animals in proportion to the natural immunity enjoyed by them. The earlier experiments of Behring appeared to give grounds for the belief that this was the case. He found, for example, that the serum of the white rat, which has a remarkable immunity to anthrax, had greater bactericidal powers than that of other animals investigated. Further investigation, however, has shown that this is not an example of a general law, and that the bactericidal action of the serum does not vary *pari passu* with the degree of immunity. In some cases non-pathogenic and also attenuated pathogenic bacteria can be seen to undergo rapid solution and disappear when placed in a drop of normal serum ; in the case of many pathogenic organisms, how-

ever, the serum has no direct bactericidal effect at all. The bactericidal action of the serum was specially studied by Nuttall, and later by Buchner and Hankin, who believed that the serum owed its power to certain substances in it, derived from the spleen, lymphatic glands, thymus, and other tissues rich in leucocytes. To these substances Buchner gave the name of *alexins*; as already explained, they correspond with Metchnikoff's cytases and Ehrlich's complements described above. They can be precipitated by ammonium sulphate and other reagents; in this respect and in their relative lability they correspond with enzymes or unorganised ferments, but in other respects, notably in their becoming rapidly used up in acting, they differ from ferments. Variations in bactericidal power of the serum as tested *in vitro*, however, do not explain the presence or absence of natural immunity against a living bacterium. In some cases, for example, it has been found to be considerable, while the organisms flourish in the body and the animal has no immunity. In such a case Metchnikoff held that there occurs in the living body no liberation of alexins by the phagocytes, and hence no bactericidal action such as occurs when the blood is shed. In the case of the hæmolytic action of a normal serum, it has been shown in many instances that, in addition to complement, a natural immune-body is also concerned (p. 200), and this would appear to be the rule; the process being analogous to what is seen in the case of an artificially developed hæmolytic serum. In certain instances an analogous condition appears to obtain in a normal bactericidal serum. For example, the dog's serum, heated at 58° C., contains a natural immune-body to B. anthracis which can be activated by the addition of normal guinea-pig's serum so as to produce a bactericidal action, though the latter is by itself without any such effect. At present, however, the possibility of bactericidal action by complement alone cannot be excluded, as it appears to combine with many bacteria without any intermediary. Further work is necessary to determine whether all the facts regarding natural immunity are explainable by the opsonic and bactericidal properties of the serum.

2. *Variations in Natural Susceptibility to Toxins.*—We must here start with the fundamental fact, incapable of explanation, that toxicity is a relative thing, or, in other words, that different animals have different degrees of resistance or non-susceptibility to toxic bodies. In every case a certain dose must be reached before effects can be observed, and up to that point the animal has resistance. This natural resistance is found to present very remarkable degrees of variation in different animals. The great

resistance of the common fowl to the toxin of the tetanus bacillus may be here mentioned (*vide* p. 534), and large amounts of this poison can be injected into the scorpion without producing any effects whatever. The high resistance of the pigeon to morphia is a striking example in the case of vegetable poisons. This variation in resistance to toxins applies also to those which produce local effects, as well as to those which cause symptoms of general poisoning. Instances of this are furnished, for example, by the vegetable poisons ricin and abrin, by the snake poisons, and by bacterial toxins such as that of diphtheria. We must take this natural resistance for granted, though it is possible that ere long it will be explained.

According to Ehrlich's view of the constitution of toxins, it might be due to the want of combining affinity between the tissue cells and the haptophore group of the toxin; or, on the other hand, supposing this affinity to exist, it might be due to an innate non-susceptibility to the action of the toxophore group. Certain investigations have been made in order to determine the combining affinity of the nervous system of the fowl with tetanus toxin, as compared with that obtaining in a susceptible animal, but the results have been somewhat contradictory. Accordingly, a general statement on this point cannot at present be made, though in all probability variations in the susceptibility to the toxophore group will be found to play a very important part. It was shown by Muir and Browning by means of hæmolytic tests that the toxic activity of complement, after it has been fixed to the corpuscles, varies very much; in some instances an amount of complement which would rapidly produce complete lysis of one kind of corpuscle, may have practically no effect on another, even though it enters into combination. These results are of importance in demonstrating how the corresponding cells of different animals may vary in sensitiveness to toxic action.

Supersensitiveness, Anaphylaxis, and Allergy

The term *supersensitiveness* is applied to states where, as compared with the normal, there is increased susceptibility or reactivity to substances introduced into the body, either parenterally or through the alimentary canal. The condition may be manifested by either local or general disturbances, and it may be natural or acquired. It has long been recognised that the ingestion of certain substances, *e.g.* shell-fish, strawberries, etc., by some normal individuals, may be followed by constitutional

disturbances, and more recently it has been found that in a small proportion of individuals the injection of a minute amount of foreign serum may give rise to such disturbances. There may be, therefore, a *natural* supersensitiveness to these substances. The increased reactivity of patients or animals suffering from an infection to the products of the infecting organism is a noteworthy example of *acquired* supersensitiveness. Thus we have the tuberculin and mallein reactions, and here both local and general effects are met with. It has come to be recognised, however, that there is a special form of supersensitiveness which is induced by the injection of substances with antigenic properties, the toxic phenomena becoming manifest only on the re-injection of the antigen after a suitable interval of time. In this form the disturbances are now known to be the result of the interaction of antibody and antigen. Thus, while the phenomena in their characters and results present a striking contrast to the state of immunity, they are in their mode of production closely related to the latter condition. To this form of supersensitiveness the term "anaphylaxis" is generally applied, and it is advisable to restrict its use in this way. The substances which have been found to have the property of calling forth the anaphylactic state—anaphylactogens—are of various kinds, including bacteria and their toxins, animal poisons, and a great many foreign proteins, *e.g.* those of serum, milk, egg albumin, etc. They are apparently all of protein nature, and they are probably coextensive with the substances which act as antigens in immunity reactions. It is important to note that many of the substances which act as anaphylactogens are on their first injection perfectly harmless.

Many of the examples of supersensitiveness, *e.g.* the tuberculin and allied reactions, hay fever, susceptibility to certain articles of diet, etc., have not yet been proved to depend on the interaction of antigen and anti-substance, that is, to be of anaphylactic nature. To this the term "allergy" (altered reactivity) may be applied, as suggested by Coca. The group is, however, not a homogeneous one, and, in most instances, the mode of production is obscure; the nature of each must be investigated separately.

Anaphylaxis.—At a comparatively early date it was found, in the case of diphtheria and tetanus toxins, that in certain instances the injection of a minute dose followed by another at a suitable interval might be attended by serious results; and that this was not an example of accumulative action, was shown by the fact that the sum of the doses might amount to only a fraction

of a lethal dose. Richet investigated a similar phenomenon in the case of a toxic substance obtained from the tentacles of actiniæ, to which, from its action, he gave the name of "congestin." He found that a certain time-interval between the injections was necessary; that after the second injection the symptoms occurred with remarkable suddenness, and that they appeared to be practically independent of the size of the first dose. He applied the term *anaphylaxis* to the supersensitive condition, and this has passed into general use; he found also that the condition lasted several weeks at least. Arthus found that, after repeated injections of horse serum in rabbits, a stage was reached at which an additional subcutaneous injection produced marked œdema and even necrosis, while an intravenous injection, harmless to an untreated animal, brought about a fatal result. The period of active research on the subject, however, may be said to date from the discovery of what is now known as the "phenomenon of Theobald Smith." This observer found that guinea-pigs which had been treated with a neutral mixture of diphtheria toxin and antitoxin might, after a certain interval of time, succumb on being injected with a quantity of normal horse serum. It was afterwards shown—especially by the researches of Otto and of Rosenau and Anderson—that the sensitising agent had really nothing to do with the toxin or antitoxin, but was due to the constituents normally present in the serum.

After this brief review we may consider some of the phenomena of anaphylaxis. In its study various sera and proteins from other sources have been employed, and the guinea-pig is the most suitable test animal; the rabbit has also been used, but its relative susceptibility is less than a hundredth of that of the guinea-pig. In the case of mice, it is difficult if not impossible to bring about serum anaphylaxis. There is, first of all, the *sensitising* injection; a guinea-pig is injected subcutaneously with a minute quantity, *e.g.* 0·001 c.c. of serum, though even 0·000001 c.c. has been found sufficient in certain instances; other methods of injection may also be employed. After a certain number of days, usually ten as a minimum, anaphylaxis has been established, and the test for this is usually made by injecting intraperitoneally 5 c.c. of the same serum, or by the intravenous route 0·5 c.c. or even less. The latter method brings about the result more rapidly. Subcutaneous injection is less effective and less certain. In the anaphylactic animal severe symptoms occur; restlessness and hyperalgesia are followed by evidence of collapse, the temperature falls markedly,

urine and fæces are passed, the heart's action becomes weak and the respiration embarrassed: in fatal cases respiration stops before the heart's action ceases. It is to be noted that the minimum amount of serum necessary to bring about the symptoms of fatal anaphylactic shock is much greater, about a thousand times greater, than the original sensitising dose; and that while anaphylaxis is not fully established till about the tenth day, it occurs gradually—not by crisis—as can be shown by disturbance of the temperature on re-injection of serum at a much earlier period. Anaphylaxis has the character of specificity, apparently within corresponding limits to immunity (p. 184)—that is, it is manifested only on the re-injection of the same protein substance as that used in the first instance. There is also a *passive* anaphylaxis, as is shown by the fact that if a certain amount of the serum of an anaphylactic guinea-pig be injected into a normal one, the latter becomes anaphylactic, so that the characteristic symptoms appear in it when the test amount of antigen (anaphylactogen) is injected. In the guinea-pig an interval of some hours at least must, however, elapse between the injections (Otto); if the two injections are made at the same time there is no result. This fact is of importance in connection with the nature and mode of causation of anaphylaxis. In the rabbit and dog, however, the symptoms appear almost at once if the two injections are given practically at the same time. Passive anaphylaxis usually disappears after a few weeks at longest, whereas active anaphylaxis has been observed after more than two years; here also there is an analogy between anaphylaxis and immunity. Another interesting observation has been made, namely, that the young of anaphylactic mothers may also be anaphylactic, and the condition may last for some time after birth.

It is also possible to produce a condition of *anti-anaphylaxis*. If, for example, the sensitising dose of horse serum is injected, and then before anaphylaxis is established (*i.e.* some time before the tenth day) another injection of a considerable quantity of serum is made, anaphylaxis does not appear, and the animal is non-susceptible to further injections of small doses for a considerable period of time. In a similar way non-susceptibility follows when an animal recovers from anaphylactic shock. On the other hand, if anaphylaxis exists, the serious effects may be avoided by the injection of a small dose of serum, insufficient in itself to bring about typical symptoms, and then by the injection of graduated increasing doses. In this instance the refractory state is brought about by a process of *desensitisation* (*vide infra*).

With regard to the symptoms and pathological changes in anaphylaxis, two facts are of importance. The first is that in a given species of animal these are of the same nature, no matter by what antigen the condition has been produced. The second is that the anaphylactic phenomena taken as a whole vary according to the species of animal. This does not mean that the condition varies essentially in nature; it probably indicates merely that different tissues are more susceptible in different animals. The symptoms of acute anaphylaxis in the guinea-pig have already been described, and the chief change found *post mortem* is an acute over-distension of the lungs which is due, as was first shown by Auer and Lewis, to a spasm of the muscle fibres in the fine bronchi and alveolar passages. In rabbits the acute symptoms are somewhat different, there being an absence of dyspnœa; the chief post-mortem change is a great accumulation of blood in the venous system, and this results from contraction of the pulmonary arterioles. In the dog there are symptoms in connection with the alimentary canal, vomiting and evacuation of the bowels, followed by a shock-like fall of the blood pressure. *Post mortem*, the chief changes are found to be a great enlargement of the liver due to accumulation of blood and an intense engorgement of the portal area. In both the rabbit and the dog there is marked leucopenia, and this is attended by diminution in the coagulability of the blood. It may be noted that in both of these animals acute anaphylaxis is less readily brought about than in the guinea-pig, and usually requires for its production more than one sensitising dose. A minor degree of sensitisation leads to a "protracted" or less acute anaphylaxis. Of acute fatal anaphylaxis in the human subject, comparatively few authentic cases have been recorded; but in one case carefully studied and described by Dean where death occurred in little more than one hour, the main symptoms and pathological changes corresponded with those observed in the dog.

Whilst it is now admitted by all that anaphylaxis depends on an interaction of antigen and anti-substance (anaphylactogen and anaphylactin), there has been much diversity of opinion as to the site and mode of its occurrence. Two chief views have been put forward. According to one, the combination takes place in the tissues and there produces disturbance; according to the other, the poison is formed by the combination in the circulating blood and thus affects various parts of the body—the *cellular* and *humoral* theories respectively. It has now been established that in an anaphylactic animal the anti-substance (anaphylactin) is present in the tissues and toxic effects result when antigen

is brought into relation with it. Schulz showed that isolated segments of the intestine of a sensitised animal responded by contraction in a specific way to the application of the antigen, and this result was confirmed by and amplified in many ways by extensive experiments by Weil and by Dale. Using the muscle of the uterus of the guinea-pig from which the blood had been removed by perfusion, Dale showed that when the specific antigen was brought into contact with it the muscle undergoes sudden contraction, which begins to pass off after a few minutes; thereafter the muscle is desensitised and does not respond further. He compared the effect to that of a powerful stimulant drug, and considered that it did not resemble enzyme action or any form of proteolysis, etc. It has been shown by various workers that the anti-substance in anaphylaxis corresponds closely in many respects with precipitin. In fact, some hold that the two substances are identical. It is thus possible that, as Dale suggests, the phenomena may be due to a disturbance of the relations of the colloids of the muscle, short of precipitation. The view as to the reaction taking place in the tissues is in harmony with other results. The fact that in an actively sensitised guinea-pig the symptoms appear at once on injection of the antigen, whereas in passive anaphylaxis a considerable time must elapse after the antiserum is injected before the animal becomes sensitive to the antigen, has always been a stumbling-block according to the theory that a poison is formed by the interaction of the two substances in the blood. It seems, however, capable of explanation on the view that the interval of time is necessary for the antibody in the serum to be fixed in the tissues. The so-called "replacement" experiments, in which the blood of the sensitised animal is replaced by normal blood, lead to a similar conclusion as to the site of the reaction; in such experiments the animal is still anaphylactic on injection of antigen and its separated tissues are so also. In addition, attempts to show that the blood of an animal in acute anaphylactic shock is toxic to another animal have given negative results.

The humoral theory that anaphylaxis is due to a toxin formed in the circulating blood by the two substances has been advanced in various forms. It was originally put forward by Richet, who called the toxic product "apotoxin." It is unnecessary to detail the various views, but reference may be made to the work of Friedberger, which included a very extensive analysis of the subject. He explained the phenomena as resulting from the process of digestion of protein introduced parenterally, the toxic

agent being a disintegration product. He showed that the action of complement on a serum precipitate (antigen+precipitin) produced a toxic body which, on being separated from the precipitate and injected into an animal, produces the symptoms of anaphylaxis ; this body he called *anaphylatoxin.* He showed also that anaphylatoxin is produced by the action of complement on bacteria treated with their antiserum, and also by the action of normal serum alone on bacteria, and even on coagulated serum. The possibility of proteolytic action by complement has, however, been called in question, and corresponding symptoms have been produced in other ways. For example, complement-containing serum on digestion with kaolin, agar, etc., has been found to acquire similar properties, and it is generally held now that the various anaphylatoxins are not specific in their action.

Dale and Kellaway have found that the effects produced by the anaphylatoxins differ from those in true anaphylactic shock, evidence of injury to the vascular endothelium and lysis of platelets being more conspicuous in the action of all of them. Further, they showed that tolerance to anaphylatoxin can be acquired and that this does not involve desensitisation of the anaphylactic animal ; on the other hand, desensitisation of an anaphylactic animal does not make it non-susceptible to the action of anaphylatoxin. The phenomena of anaphylaxis, however, are very complicated, and whilst the combination of antigen with antibody in the tissues leads to the essential effects, the possibility that toxic bodies formed in the blood itself may play a part cannot be excluded ; this is more likely to be the case when death does not occur quickly. In anaphylaxis there is often a marked fall in complement, and this is apparently due to its fixation in the usual way by the combination of antigen+anti-substance. There is evidence that the diminution in the coagulability of the blood, which is a noteworthy change in the rabbit and the dog, results from the setting free of anti-coagulants from the liver. The marked leucopenia, which is often a striking feature, is due to the accumulation of the leucocytes in the lungs and other organs, in a similar manner to what is seen after the injection of Witte's peptone or other substances into the blood.

Besredka considers that the sensitising and the toxic factors in the horse serum are not one and the same. He finds that serum heated to a certain temperature may still have the power of inducing the condition of anaphylaxis, but has lost the power of bringing about the toxic phenomena when injected into an anaphylactic animal. This result has, however, been explained by others as

being due to the fact that the sensitising dose is so much smaller than the toxic dose (*vide supra*) on re-injection ; accordingly, the effect of heat may be to reduce the latter below the fatal limit without having a corresponding effect on the sensitising dose.

Allergy.—It is still an open question as to what extent the phenomena of anaphylaxis just described are of the same nature as the supersensitiveness or allergy manifested by patients suffering from disease to the products of the corresponding organism, *e.g.*, to tuberculin, mallein, etc. (pp. 335, 360); though in all probability they are at least similar in essence. It was held for some time as a distinction that this supersensitiveness in infections to bacterial products could not be transferred to another animal, but recent observations show that in certain circumstances this is possible in the case of tuberculin. According to one view, which appears to us to have most in support of it, the phenomena of supersensitiveness of tubercular patients to tuberculin is due to the combination of the injected antigen with molecules of antibody resident in the tissue cells, the so-called " sessile receptors." Friedberger, however, holds that the facts can be equally well explained by the combination, which occurs either locally or generally, of the antigen with antibody *in the serum*, which combination when acted upon by complement gives rise to the poisonous substance. Others, again, consider that there is not sufficient evidence that an antibody is concerned, and that local allergy is of a different nature. At present it is not possible to make a definite statement on the subject. Further details are given in connection with the special chapters. There is no doubt that the supersensitive condition must play an important part in the clinical manifestations of many diseases. For example, the sensitiveness of tubercular patients to tuberculin shows that the symptoms in this disease are evidently produced by the absorption from the tubercular foci of a smaller amount of toxin than would be necessary to produce corresponding effects in a normal individual. And the sensitiveness of the conjunctiva in typhoid fever to the products of the bacillus suggests that in this disease also supersensitiveness plays an important part.

It appears at least likely that bacterial products set free in the body in an infection gradually produce a state of supersensitiveness to these products, which is closely similar to allergy if not to a true anaphylactic state. The phenomena of hay fever probably belong to the same class, being apparently the result of repeated absorption of vegetable proteins ; a similar statement may apply to the sensitiveness to articles of diet. In all

such cases, however, the possibility of a natural supersensitiveness as an idiosyncrasy must be recognised. Cases of supersensitiveness of children to cow's milk have been recorded, and it is possible that such may be due to the absorption from the alimentary tract of protein molecules, or, at least, of derivatives which may act as antigens. The possibility of such an occurrence is shown by the work of Ehrlich on the vegetable toxins, ricin and abrin (p. 183). A certain amount of evidence has been brought forward that puerperal eclampsia is produced by the absorption of proteins from the placenta, which have the property of establishing an anaphylactic state. It must be admitted however, that our knowledge as to such problems is still very defective.

The Serum Disease in Man.—Apart from acute anaphylaxis in man referred to above, symptoms of milder degree are not infrequently observed after the injection of serum for therapeutic purposes. They were first fully described as a syndrome by v. Pirquet and Schick, and the following may be given as a summary. There is here also a period of incubation, of eight to twenty days on the average ; after which, in a certain proportion of cases (in about 20 per cent.) on the injection of a fairly large amount of horse serum, a group of characteristic symptoms appear. There may be as prodromal symptoms, swelling and tenderness at the site of injection, and in the corresponding lymphatic glands, and thereafter general exanthemata appear. These are usually of an urticarial type, but may be erythematous or morbilliform. There is usually moderate pyrexia of a remittent type, and sometimes œdema and slight albuminuria are present ; occasionally there are pains in the joints ; there is also often leucopenia, due to a fall in the number of polymorpho-nuclear leucocytes. These symptoms last for a few days and then disappear. Such are the phenomena of the serum disease after a single injection of the foreign serum. There are, however, two other types of reaction described by v. Pirquet and Schick, namely, the *immediate* and the *accelerated* reactions. The immediate reaction is seen when a large dose of serum has been administered, and then after a certain interval of time another dose of serum is injected. This interval is usually from twelve days to eight weeks, though sometimes as long as six months. The symptoms of the immediate reaction, which appear shortly after the injection, or at least within twenty-four hours, are an intense œdema locally, general exanthemata and pyrexia, though the general phenomena are often little marked. The symptoms pass off comparatively quickly, usually within

twenty-four hours. The accelerated reaction is also seen after a second injection, and it may occur from six weeks up to many months after the first injection. In the case of the accelerated reaction there is an incubation period, but it is shorter than in the case of the first injection, being usually from five to seven days ; the symptoms resemble those in the ordinary reaction as described above, but are of rather more acute onset and last a shorter time. In the interval from about the sixth week to the sixth month, there may occur both the immediate reaction, and also a few days later an accelerated reaction.

The phenomena of the serum disease in all probability depend upon the development of a reaction-body or antibody, as above described, though this view has been called in question by some writers. Cases are recorded where symptoms have occurred almost at once following the first injection, or after a time too short to correspond to a true incubation period. These may, however, be examples of natural hypersensitiveness. But the facts described above, especially with regard to the phenomena after a second injection, seem to point strongly to an antibody being concerned in the reaction. In recent times the intracutaneous injection of a small quantity of the serum has been used as a test for supersensitiveness (*vide infra*), and this apparently depends on an antigen+antibody reaction.

Practical Results—Desensitisation.—In view of the common use of curative serum, anaphylaxis has come to have considerable practical importance, especially in connection with intravenous injection, as by this route the dangerous dose is a fraction of that by subcutaneous injection. With regard to the possibility of there being a primary or *natural* supersensitiveness, inquiry should be made as to tendency to asthma or hay fever, or sensitiveness to the presence of horses in the vicinity, as these have been found to be associated conditions, and the existence of Graves' disease has been recorded as another. Then with regard to the *acquired* variety, information should be obtained as far as possible regarding previous serum injections. The existence of supersensitiveness can, however, be demonstrated by the test for skin "allergy." A small quantity, say 0·1 c.c., of sterile horse serum is injected by a hypodermic needle into the dermis—not subcutaneously. The minute local swelling which results from the presence of the fluid soon passes off. But in the case of a positive reaction there occurs, usually within five to thirty minutes, an urticarial patch, which may be followed by a distinct vesicle and is often surrounded by an erythematous area, an inch or more in diameter. If no reaction occurs within

forty minutes the absence of supersensitiveness may be inferred. If a positive reaction is obtained, means must be taken to desensitise the patient, *i.e.*, to produce anti-anaphylaxis ; and this is accomplished by introducing initial small doses of serum and then gradually increasing them. Even then, care should be used in injecting the serum intravenously, as desensitisation in the human subject is sometimes uncertain.

In the hospital of the Rockefeller Institute, where large intravenous doses of serum are given in the treatment of pneumonia, the initial desensitising dose is 0·025 c.c. given subcutaneously, and this amount is doubled every half-hour. If no reaction follows the administration of 1 c.c., the subsequent doses are given intravenously, commencing with 0·1 c.c. and doubling the dose every half-hour till 25 c.c. in all have been given in these small doses. Such a method, however, takes a considerable number of hours and is not justifiable in a case of tetanus, where a large amount of serum should be given intravenously or intrathecally as soon as possible. The following method, given in the War Office memorrandum, should be followed : 5 c.c. of the antiserum are diluted with 50 c.c. of normal salt solution. Of the mixture 1 c.c. is injected intravenously ; this is followed four minutes later by 3 c.c., two minutes later by 10 c.c., and two minutes later again by 25 c.c. Then after ten to fifteen minutes the full dose may be given intravenously or intrathecally. The doses mentioned are most suitably given by the gravitation method.

If any anaphylactic symptoms appear, the administration must be temporarily stopped and then cautiously resumed. The chief symptoms are dyspnœa, with pallor or cyanosis, fall in the blood pressure, with feeble pulse, asthmatic symptoms, with cough, and sometimes vomiting. Adrenalin and atropine are the most efficient drugs. In all cases the administration of serum by the methods mentioned should be carried out slowly and with caution. Anaphylaxis is sometimes a real danger, but the risks, when we take into account the necessity for the prompt treatment of tetanus, have been exaggerated. We may add that the repeated subcutaneous injections for preventive purposes of the usual quantity of 3 c.c. of serum are unattended by any danger. It may also be stated that in relation to anaphylaxis it is only the *amount of serum* and the *species* from which it is drawn which matter—the antitoxic value is not a factor.

CHAPTER VII

INFLAMMATORY AND SUPPURATIVE CONDITIONS

THIS subject is an exceedingly wide one, and embraces a great many pathological conditions which in their general characters and results are widely different. Thus, in addition to suppuration, various inflammations, endocarditis, septicæmia, and pyæmia, will come up for consideration. With regard to these, the two following general statements, established by bacteriological research, may be made in introducing the subject. In the first place, there is no one specific organism for any of these conditions; various organisms may produce them, and not infrequently more than one organism may be present. In the second place, the same organism may produce widely varying results under different circumstances—at one time a local inflammation or abscess, at another multiple suppurations or a general septicæmia. The principles on which this diversity in results depends have already been explained (p. 150). Furthermore, there are conditions like acute pneumonia, epidemic meningitis, acute rheumatism, etc., which have practically the character of specific diseases, and yet which, as regards their essential pathology, belong to the same class. Strong evidence has also been brought forward by recent work that scarlatina, a specific infectious disease, is due to a hæmolytic streptococcus, that is, a type of organism commonly associated with inflammatory lesions. The arrangement followed below is to a certain extent one of convenience.

It may be well to emphasise some of the chief points in the pathology of these conditions. In *suppuration* the two main phenomena are—(*a*) a progressive emigration of leucocytes, chiefly of the polymorpho-nuclear (neutrophile) variety, and (*b*) a liquefaction or digestion of the supporting elements of the tissue along with necrosis of the cells of the part. The result is that the tissue affected becomes replaced by the cream-like fluid called pus. A suppurative inflammation is thus to be distinguished on the one hand from an inflammation without

destruction of tissue, and on the other from necrosis or death *en masse*, where the tissue is not liquefied, and leucocyte accumulation may be slight. When, however, suppuration is taking place in a very dense fibrous tissue, liquefaction may be incomplete, and a portion of dead tissue or slough may remain in the centre, as is the case in boils. In the case of suppuration in a serous cavity the two chief factors are the progressive leucocytic accumulation and the disappearance of any fibrin which may be present.

Many experiments have been performed to determine whether suppuration can be produced in the absence of micro-organisms by various chemical substances, such as croton oil, nitrate of silver, turpentine, etc.—care, of course, being taken to ensure the absence of bacteria. The general result obtained by independent observers is that, as a rule, suppuration does not follow, but that in certain animals and with certain substances it may, the pus being free from bacteria. Buchner showed that suppuration may be produced by the injection of dead bacteria, *e.g.*, sterilised cultures of Bacillus pyocyaneus, etc., and, according to Vaughan, bacterial protein *per se* may produce suppuration and necrosis of tissue. The subject has now more a scientific than a practical interest, and the general statement may be made that practically all cases of true suppuration met with clinically are due to the action of living micro-organisms.

The term *septicæmia* is applied to conditions in which the organisms multiply within the blood and give rise to symptoms of general poisoning, without, however, producing abscesses in the organs. The organisms are usually more numerous in the capillaries of internal organs than in the peripheral circulation, but the application of methods of blood culture has shown that they can be detected in the peripheral blood much more frequently than was formerly supposed to be the case. The essential fact in *pyæmia*, on the other hand, is the occurrence of multiple abscesses in internal organs and other parts of the body. In most of the cases of typical pyæmia, common in pre-antiseptic days, the starting-point of the disease was a septic wound with bacterial invasion of a vein, leading to thrombosis and secondary embolism. Multiple foci of suppuration may be produced, however, in other ways, as will be described below (p. 254). If the term "pyæmia" be used to embrace all such conditions, the method of their production should always be distinguished.

Bacteria as Causes of Inflammation and Suppuration

A considerable number of species of bacteria have been found in acute inflammatory and suppurative conditions, and most of these have been proved to be causally related. It should be recognised that many organisms when experimentally introduced into the tissues are capable of producing an acute inflammation and even pus formation ; but only certain species are characteristically associated with such lesions under natural conditions. These organisms now to be described are usually known as *pyogenic.*

Ogston, who was one of the first to study this question (in 1881), found that the organisms most frequently present were micrococci, of which some were arranged irregularly in clusters (staphylococci), whilst others formed chains (streptococci). He found that the former were more common in circumscribed acute abscesses, the latter in spreading suppurative conditions. Rosenbach shortly afterwards (1884), by means of cultures, differentiated several varieties of micrococci, to which he gave the following special names : *Staphylococcus pyogenes aureus, Staphylococcus pyogenes albus, Streptococcus pyogenes,* and an organism called by him *Micrococcus pyogenes tenuis.* Suppuration may be produced, however, by various other species, *e.g.* different types of staphylococci and streptococci (*vide infra*), *Micrococcus tetragenus, Pneumococcus, Meningococcus, Gonococcus, bacteria of the Bacillus coli group, Pneumobacillus* (Friedländer), *Bacillus pyocyaneus, Bacillus proteus, Actinomyces, Bacillus mallei,* etc. Even the typhoid and paratyphoid bacilli, which primarily are not pyogenic organisms, may be associated with suppurative lesions occurring as complications or sequelæ of enteric fever. The bacillus of influenza may also occur in secondary inflammatory and suppurative conditions. Various anaerobic bacteria are also concerned in the production of an inflammation which is often associated with œdema, hæmorrhage, or necrosis (*vide* Chapter XXI.).

Staphylococcus pyogenes aureus (*Staphylococcus aureus, Rosenbach*).—*Microscopical Characters.*—This organism is a spherical coccus about 0·9 μ in diameter, which tends to grow in irregular clusters or masses (Fig. 43). It stains readily with all the basic aniline dyes, and retains the colour in Gram's method (Plate I., Fig. 1).

Cultivation.—Growth occurs under aerobic conditions, but the organism is also a facultative anaerobe. It grows readily

in all the ordinary media at the room temperature, though much more rapidly at the temperature of the body. On *agar*, a stroke culture forms a line of abundant yellowish growth, with smooth shining surface, well formed after twenty-four hours at 37° C. Later it becomes bright orange in colour, and resembles a streak

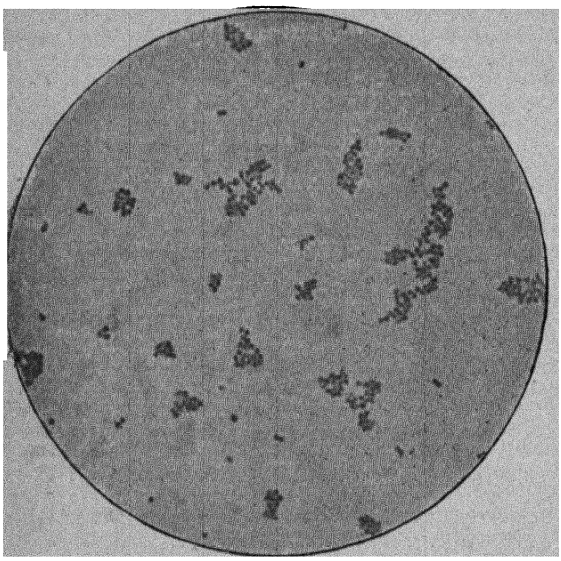


FIG. 43. — Staphylococcus pyogenes aureus, young culture on agar, showing clumps of cocci. Stained with weak carbol-fuchsin. ×1000.

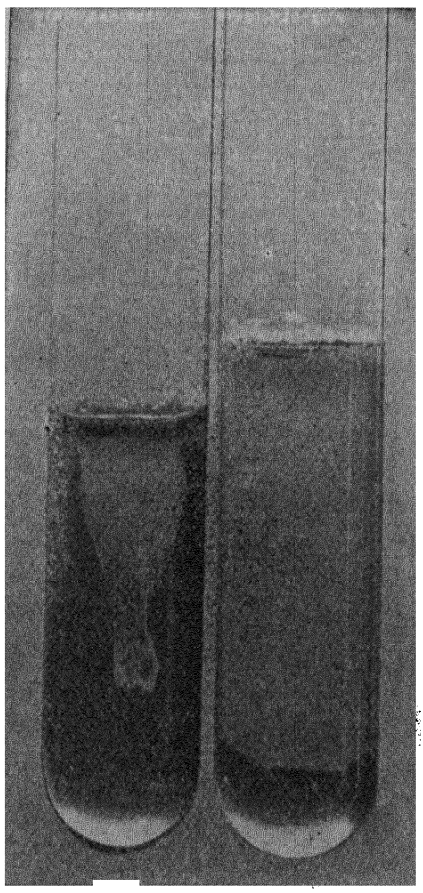


FIG. 44.—Two stab cultures of Staphylococcus pyogenes aureus in gelatin—(*a*) 10 days old; (*b*) 3 weeks old, showing liquefaction of the medium and characters of growth. Natural size.

of oil paint. Single colonies on the surface of agar are circular discs of similar appearance, which may reach 2–3 mm. after twenty-four to forty-eight hours. On blood agar a clear zone of hæmolysis is noted round colonies due to the diffusible hæmolysin produced by the organism. In stab cultures in *peptone gelatin* a streak of growth is visible on the day after inoculation, and on the second or third day liquefaction commences at the top. As liquefaction proceeds, the growth falls to the bottom as a flocculent deposit, which soon assumes a bright yellow colour, while a yellowish film may form on the surface, the fluid portion still remaining turbid. Ultimately liquefaction extends out to the wall of the tube (Fig. 44). In *gelatin plates* colonies may be seen with the low power of the microscope in twenty-four hours, as little balls somewhat granular on the surface and of

brownish colour. On the second day they are visible to the naked eye as whitish-yellow points, which in typical strains afterwards become more distinctly yellow. Liquefaction occurs around these, and little cups are formed, at the bottom of which the colonies form yellowish masses. On *solidified serum*, liquefaction occurs as in the case of a gelatin medium. On *potato* it grows well at ordinary temperature, forming a somewhat abundant layer of orange colour. In *bouillon* it produces a uniform turbidity, which afterwards settles to the bottom as an abundant layer and assumes a brownish-yellow tint. In the various media it renders the reaction acid, and coagulates milk, in which it readily grows. The cultures have a somewhat sour odour. It has considerable tenacity of life outside the body, and withstands drying. The thermal death-point is approximately 62° C., but some strains resist higher temperatures, even 70° C. for half an hour.

The Staphylococcus Pyogenes Albus (*Staphylococcus albus, Rosenbach*) is similar in character, with the exception that its growth on all the media is white. The colour of the staphylococcus aureus may develop slowly or may become less distinctly yellow after being kept for some time in culture, but it never assumes the white colour of the staphylococcus albus, and it has not been found possible to transform the one organism into the other. A similar organism, called by Welch *Staphylococcus epidermidis albus*, is practically always present on the skin it is distinguished by its relatively non-pathogenic properties, by liquefying gelatin somewhat slowly, and by being non-hæmolytic. It is probably an attenuated variety of the staphylococcus pyogenes albus.

The *Staphylococcus pyogenes citreus*, which is less frequently met with, differs in the colour of the cultures, being a lemon-yellow, and is usually less virulent than the other two.

Other varieties of staphylococci have also been described, Staph. cereus flavus and Staph cereus albus, so designated in virtue of their wax-like growth, and the coloration produced. They do not liquefy gelatin. These types are of rare occurrence.

Staphylococci occur as normal commensal organisms on the skin, in the mouth, throat, and nose, the prevalent type being the staphylococcus albus. These organisms are therefore found in the air, in dust, on clothing, etc. Staphylococci have also been found in a latent state in the tonsils and in lymphatic glands.

Streptococcus pyogenes (*Streptococcus pyogenes, Rosenbach*).—This organism (Plate I., Fig. 1) is a coccus of slightly larger size

than the staphylococcus aureus, about 1 μ in diameter, and forms chains which may contain a large number of cocci, especially when it is growing in fluids (Fig. 45). The chains vary somewhat in length, depending largely on the environment. As division may take place in many of the cocci in a chain at the same time, the appearance of a chain of oval forms and diplococci is often met with. In young cultures the cocci are fairly uniform in size, but after a time they present considerable variations, many swelling up to twice their normal diameter. These are to

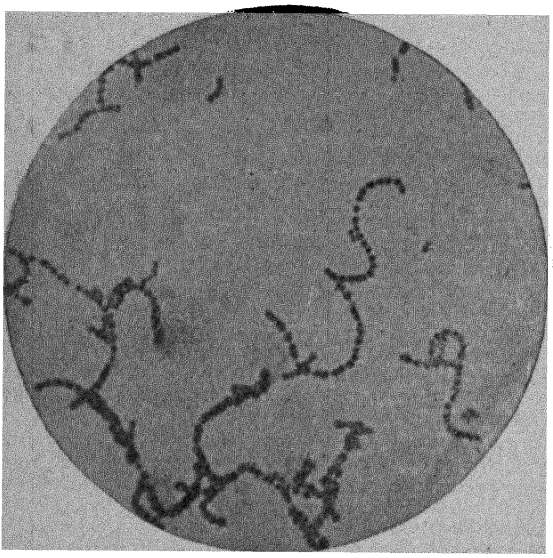


FIG. 45.—Streptococcus pyogenes, young culture on agar, showing chains of cocci.
Stained with weak carbol-fuchsin.
× 1000.

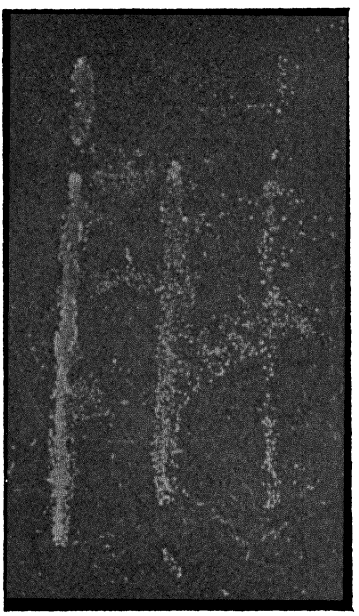


FIG. 46.—Culture of the Streptococcus pyogenes on an agar plate, showing numerous colonies—three successive strokes. Twenty-four hours' growth. Natural size.

be regarded as involution forms. In its staining reactions the streptococcus resembles the staphylococci described, being stained positively by Gram's method.

Cultivation.—In cultures outside the body the streptococcus pyogenes grows much more slowly than the staphylococci, and also dies out more readily, being in every respect a more delicate organism. It grows aerobically on the ordinary culture media, *e.g.* nutrient agar, the optimum temperature being about 37° C. Most strains will not grow at room temperature. The addition of blood or serum to the medium enhances growth, and blood agar is a convenient medium for routine cultivation (*vide infra*).

On the *agar* media, growth takes place along the stroke as a collection of small circular discs of semi-translucent appearance, which show a great tendency to remain separate (Fig. 46). The separate colonies remain small, rarely exceeding 1 mm. in diameter. Under a low power of the microscope they have a slightly woolly margin. Cultures on agar kept at the body temperature may often be found to be dead after ten days. On *blood agar*, the colonies are surrounded by a clear zone of laking or hæmolysis due to the hæmolysin produced by the organism. This presents a striking cultural character. In *peptone gelatin*, if growth occurs at low temperature, a stab culture shows about the second day a thin line, which in its subsequent growth is seen to be formed of a row of minute rounded colonies of whitish colour; these may be separate at the lower part of the puncture. They do not usually exceed the size of a small pin's head, this size being reached about the fifth or sixth day. The growth does not spread on the surface, and no liquefaction of the medium occurs. In *milk* it produces a strongly acid reaction but no clotting of the medium. It ferments glucose, lactose, saccharose, and salicin (Andrewes and Horder); it produces no fermentation of inulin, in this respect differing from the pneumococcus (p. 272). In *bouillon*, growth forms numerous minute granules which afterwards fall to the bottom, the deposit, which is usually not very abundant, having a sandy appearance. The appearance in broth, however, presents variations. Certain strains forming in fluid media long chains, which become wound up in the form of spherules ("*S. conglomeratus*"), develop a markedly granular growth in bouillon.

Varieties of Streptococci.—Formerly the *Streptococcus pyogenes* and the "*Streptococcus erysipelatis*" were regarded as two distinct species, and various points of difference between them were given. Further study, and especially the results obtained by modifying the virulence (p. 248), have shown that these distinctions cannot be maintained, and now practically all authorities are agreed that the two organisms are one and the same, erysipelas being produced when the streptococcus pyogenes of a certain standard of virulence gains entrance to the lymphatics of the skin. Petruschky, moreover, showed conclusively by inoculation that a streptococcus cultivated from pus could cause erysipelas in the human subject.

Streptococci have also been classified according to the length of the chains. Thus there have been distinguished (*a*) *Streptococcus longus*, which occurs in long chains and is pathogenic to rabbits and mice; (*b*) *Streptococcus brevis*, which is common in

the mouth in normal conditions, and is usually non-pathogenic; and (*c*) *Streptococcus conglomeratus*, so called from its forming in bouillon minute granules composed of very long chains. French writers first described a short-chained variety under the title *Enterococcus*; this for convenience will be described separately. It may be stated that pathogenic streptococci obtained from the human subject usually form fairly long chains, whilst the short streptococci obtained from the mouth and intestine are *usually* devoid of virulence. But to these statements exceptions occur, as short streptococci may be associated with grave lesions, and long streptococci without virulence may be obtained on normal mucous membranes; in view of all the facts, pathogenicity and morphology cannot be taken as affording in themselves a basis of classification. Anaerobic streptococci have been isolated from war wounds (Fleming); they have also been described by Prévot in gangrene of the lung, uterus, and appendix. Accordingly, other methods have been introduced as a means of differentiation, and of these the most important are fermentation and hæmolytic tests.

Fermentation.—Mervyn Gordon introduced for this purpose nine tests, namely: (1) The clotting of milk, (2) the reduction of neutral-red, (3–9) the fermentation with acid production of saccharose, lactose, raffinose, inulin, salicin, coniferin, and mannite. Andrewes and Horder by means of these have differentiated six varieties, of which five occur in the human subject. These are: (*a*) A short-chained form called *Streptococcus mitis*, which occurs chiefly in the saliva and fæces as a saprophyte. It ferments saccharose and lactose, and sometimes the glucosides; it produces an acid reaction in milk but no clotting, and often reduces neutral-red. (*b*) The *Streptococcus pyogenes*, which is the most important pathogenic variety, and has the characters described above. (*c*) The *Streptococcus salivarius*, which corresponds to the streptococcus brevis of the mouth, and which, as regards fermentative action, seems to bear the same relation to the next variety as the streptococcus mitis does to the streptococcus pyogenes. It ferments saccharose, lactose, and raffinose, sometimes the glucosides and rarely inulin; it clots milk and reduces neutral-red. (*d*) The *Streptococcus anginosus*, which corresponds with the so-called streptococcus conglomeratus. It ferments saccharose and lactose, and sometimes raffinose, reduces neutral-red, and is actively hæmolytic. It usually clots milk and does not grow on gelatin at 20° C. (*e*) The *Streptococcus fæcalis*, a short-chained form, which abounds in the intestine and which has great fermentative activity, and reacts positively to all Gordon's tests with the exception of raffinose and inulin. It forms sulphuretted hydrogen, and is devoid of hæmolytic action. (*f*) The sixth variety is the *Streptococcus equinus*, which is common in the air and dust of towns, and appears to be derived from horse dung. It ferments saccharose and the two glucosides, and forms little or no acid in milk. It is, however, to be noted that to all these types variants are met with.

Ainley Walker in studying this question, however, found that various strains of streptococci show considerable variability in their fermentative powers when kept for some time under ordinary conditions of growth, and Beattie and Yates have observed corresponding changes when streptococci are passed through the animal body. Nevertheless fermentative activity has been generally accepted as of great service in classification, especially when the organisms have been recently isolated.

Hæmolysis (*vide* also p. 243) —Schottmüller has employed the appearance of the colonies of streptococci on blood agar as a means of separating varieties. The medium used by him consisted of 2 parts human blood (rabbit blood may likewise be used) and 5 parts melted agar; it is, however, better to add the blood in the proportion of 5–10 per cent. He distinguished the *Streptococcus longus* or *erysipelatis*, which forms grey colonies and has a marked hæmolytic action; a *Streptococcus mitior* or *viridans*, a short-chained organism, which produces small colonies of green colour, ascribed to production of methæmoglobin, and very little hæmolysis; and a *Streptococcus mucosus encapsulatus*, which, as its name indicates, shows well-marked capsules and produces colonies which have a slimy consistence. Mandelbaum adds to these the *Streptococcus saprophyticus*, which is without hæmolytic action. (It should be noted that on blood agar the pneumococcus forms green colonies and produces little or no hæmolysis.) Smith and Brown have designated the so-called hæmolytic streptococci as type β, the viridans strains as the type α. They regard the type α as weakly hæmolytic and draw attention to the partial hæmolysis in the discoloured zone round colonies on blood agar. The streptococcus saprophyticus of Mandelbaum is their type γ; it is entirely devoid of hæmolytic properties and causes no discoloration on a blood medium. Levy found that a 2·5 per cent. solution of taurocholate of sodium in bouillon produced complete bacteriolysis of the pneumococcus and the streptococcus mucosus, while it had no effect on other varieties of streptococcus. He considered the streptococcus mucosus to be a variety of pneumococcus. On the other hand, some strains of streptococcus mucosus have been found to be insoluble in bile-salts; hence two varieties have been distinguished (*vide* p. 273). The general statement may be made that most of the streptococci from suppurative lesions in the human subject have hæmolytic action, but that occasionally streptococci without this property are found even in severe infections. Marked alterations in morphological and biological characters of organisms belonging to the streptococcus group may occur in the animal body. Thus Bordet pointed out that development of a capsule *in vivo* accompanied increase in virulence; Schnitzer and Munter found, after inoculation with a culture of hæmolytic streptococci, that a proportion of the organisms recovered a few hours later were non-hæmolytic and reduced in virulence.

A combination of fermentative and hæmolytic tests has also been used in classification, and this appears advisable. For example, Holman takes the presence or absence of hæmolysis as the first basis of classification. The two groups thus obtained are subdivided into four, according to the action on lactose; these again into eight, according to the action on mannite; and finally into sixteen, according to the effect on salicin. Blake employs the

same principle but omits mannite, and does not subdivide the hæmolytic group.

Gordon (1922) has summarised as follows the chief features of the three most important streptococci :

	Hæmolysis.	Raffinose.	Mannite.
Str. pyogenes	+	−	−
Str. salivarius	−	+	−
Str. fæcalis.	−	−	+

Enterococcus. — This variety, when growing in the body, usually occurs as a diplococcus, the individual organisms being rounded or oval, and the members of a pair being often set at an angle, and unequal in size ; sometimes there is indication of a capsule. In cultures it shows considerable pleomorphism, and tends to grow in masses, though short chains occur in fluid media. On the surface of agar it produces a thin, semi-transparent layer with smooth margins, and there is not the tendency to form separate colonies which is shown by most streptococci. In a corresponding way it forms a diffuse turbidity in bouillon, with the formation after a time of a somewhat glairy deposit ; sometimes there is a scum on the surface. It flourishes well at a lower temperature than that at which the streptococcus pyogenes will grow, and has great longevity in cultures. It possesses a relatively high resistance to heat—a point of importance. Broth cultures survive exposure to 60° C. for fifteen minutes, while other varieties of streptococci are killed in five to ten minutes at this temperature. When first isolated, some strains have been found to prefer anaerobic conditions. It has very active fermentative properties and coagulates milk ; it is closely related to the streptococcus fæcalis described above. Some strains have been found to liquefy gelatin. It is non-hæmolytic and relatively non-virulent ; in fact most strains can be injected in large doses without pathogenic effects. It is a normal inhabitant of the intestine, and has been found to appear in the intestine of the infant shortly after birth. The lesions in which it is found are chiefly those where infection can be traced from the bowel. It is practically always present in contaminated gun-shot wounds at an early stage (*vide* p. 249), gradually disappearing at a later stage. It has been found in abscesses following typhoid, and not infrequently in the bladder during or after bowel infections, though also apart from these. It was obtained during the war from the blood and bladder in a fair proportion of cases of septicæmic type, in others where myalgia was the chief feature, in others again of the type of " trench fever " (Houston and McCloy).

On the whole, there may be said to be substantial agreement in the results of those who have systematically used fermentation tests. In the description of any strain these should be taken

along with other characters—morphology, growth conditions, pathogenicity, hæmolytic effects, solubility or non-solubility in bile-salts, etc.

Serological Classification of Streptococci.—Attempts have been made to classify streptococci by serological methods, mainly by agglutination with specific antisera, correlated with agglutinin absorption tests. Agglutination tests with streptococcus cultures present a certain amount of technical difficulty, in view of the tendency to auto-agglutination or the granularity of the suspension prepared from the usual cultures. It has been found that cultures in veal broth containing 0·2 per cent. disodium hydrogen phosphate, 0·1 per cent. glucose, and 1 per cent. peptone standardised to $P_H = 7·5$, prove most suitable for providing a uniform suspension for agglutination tests. The growth is deposited by centrifuging, the supernatant fluid is removed and a suspension is prepared by suspending the growth in saline. A strain that tends to produce a granular growth in fluid medium or a granular suspension, and is therefore unsuitable for agglutination tests, should be repeatedly subcultured in the phosphate broth until a uniform growth is obtained.

Serologically, streptococci appear to represent a heterogeneous group. The hæmolytic sub-group has been regarded by some observers as homogeneous by precipitin tests (Hitchcock) and by complement fixation tests (Clawson). Dochez, Avery, and Lancefield claimed that the hæmolytic streptococci could be classified as six serological types by agglutination reactions; and Bliss found that one of these types included the scarlet fever strains. Gordon recognised three serological types of hæmolytic streptococci—I. S. pyogenes (the largest), II. (rare), and III. S. scarlatinæ (*vide infra*). According to Eagles, the scarlet fever strains form a clear-cut serological group and the same applies to puerperal sepsis strains. Even among the scarlatina strains, however, serological heterogeneity has been observed by different workers (*vide infra*). It has been shown by Norton, who used the agglutinin absorption test, that the viridans group is also heterogeneous. It is uncertain whether any definite classification of the streptococci can be based on serological characters.

Toxins of Pyococci.—As stated above, many streptococci have a distinct hæmolytic action, and this is due to the production of a toxin which is largely extra-cellular. The amount of hæmolysin formed varies greatly in the case of different strains and also according to the medium used. M'Leod recommends a medium composed of 20 per cent. horse serum and 80 per cent. peptone bouillon with distinctly alkaline reaction, and has found a Maassen filter to be specially suitable for obtaining the hæmolytic filtrate. In the medium mentioned the maximum formation of hæmolysin is reached in about eighteen hours, and thereafter a diminution occurs. The hæmolysin is very labile, being destroyed at 55° C., and rapidly deteriorating even when

kept in the incubator for a few hours. The filtrate has also a toxic action on the tissues, producing focal necrosis especially in the liver of the rabbit. It is also a noteworthy fact that an antitoxin to the hæmolysin cannot be obtained. Streptococcus viridans, though producing little or no lysis, has the property of forming methæmoglobin from hæmoglobin. The staphylococcus aureus and staphylococcus albus also produce hæmolysins, which so far as can be judged by their properties are identical. (The staphylococcus epidermidis albus, however, produces no hæmolysin.) The staphylolysin, which can readily be obtained by filtering alkaline broth cultures after incubation at 37° C. for ten to fourteen days, though more stable than the streptolysin, is also destroyed at a temperature of 55° C. It, however, differs from the latter, inasmuch as an antitoxin can readily be obtained to it; in fact, in its properties it presents a close analogy to the toxins of diphtheria and tetanus. The two staphylococci mentioned also produce a toxin which kills leucocytes, and is therefore called "leucocidin" (van de Velde). This toxin can be obtained by filtration of fluid cultures, and on being injected into animals leads to the formation of an antitoxin. Apparently the same leucocidin is produced by the staphylococcus aureus and staphylococcus albus. The apparently specific toxin of Str. scarlatinæ is referred to on p. 264.

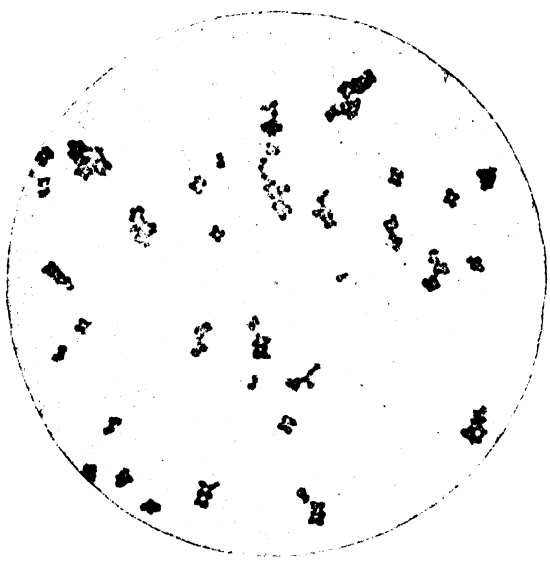


FIG. 47.—Micrococcus tetragenus; young culture on agar, showing tetrads. Stained with weak carbol-fuchsin. ×1000.

Immunity.—The chief phenomena have been dealt with in Chapter VI.—for example, opsonic action and the principles of vaccine treatment (pp. 201, 182), anti-streptococcic serum (p. 195), local immunity (p. 183).

Micrococcus tetragenus (*Staphylococcus tetragenus, Koch-Gaffky*).—This organism, first described by Gaffky, is characterised by the fact that it divides in two planes at right angles to one another (Fig. 47), and is thus generally found in the tissues in groups of four, or tetrads, which are often seen to be surrounded by a capsule.

The cocci measure about 1 μ in diameter. They stain readily with all the ordinary stains, and also retain the stain in Gram's method.

It grows readily in all the media at the room temperature. In a puncture culture in peptone-gelatin a fairly thick whitish line forms along the track of the needle, whilst on the surface there is a thick rounded disc of whitish colour. The gelatin is not liquefied. On the surface of agar and of potato the growth is an abundant moist layer of the same colour. The growth on all the media may have a viscid character.

White mice are exceedingly susceptible to this organism when recently isolated. Subcutaneous injection is followed by a general septicæmia, the organism being found in large numbers in the blood throughout the body. Guinea-pigs are less susceptible; sometimes only a local abscess with a good deal of necrotic change results; sometimes there is also septicæmia.

Bacillus coli (*Escherichia coli, etc.*) —The microscopic and cultural characters of organisms of this group, which may be associated with inflammatory and suppurative lesions, *e.g.* of the urinary system, are described in a later chapter.

Bacillus proteus. —The term *proteus* has been applied to a group of intestinal bacteria, of which several varieties have been described, *e g vulgaris, mirabilis, zenkeri, capsulatus*; the "*Urobacillus septicus*" is also a variety. They are characterised by their pleomorphism, hence the name, and by their rapid liquefaction of gelatin. B. proteus has the following characters: It is a small bacillus of about the size of B. coli, straight or slightly curved, but coccoid and filamentous forms also occur, and a marked tendency to involution forms is to be noted. It is actively motile, and possesses numerous lateral flagella; it does not form spores. It stains readily with basic dyes and is Gram-negative. It grows readily on all the ordinary media at room temperature, but best at the body temperature. On an agar slope the organism forms a moist layer, which extends over the whole surface of the medium; in this way the bacillus can readily be separated from other organisms present along with it, but in mixed culture other organisms are difficult to separate from it owing to its spreading growth. This type of organism is therefore spoken of as a "spreader"; the same spreading growth is noted in plate cultures but is not invariably present. In a gelatin stab culture liquefaction appears within twenty-four hours; it spreads rapidly in the form of a funnel, and ultimately the whole medium is liquefied and presents a turbid appearance. In gelatin plates the characters are somewhat peculiar, especially when 5 per cent. gelatin is used. The colonies are at first small spheres with granular centre and peripheral radiation extending into the medium; liquefaction soon follows, and from the superficial colonies offshoots extend over the medium in tendril-like fashion, these being composed of bacilli in chains placed side by side. Groups of bacilli may also become separate, move over the surface of the medium, and form growths at a distance—the so-called "swarm-colonies." On potato it forms a slimy film with some discoloration around it. It coagulates milk without acid reaction. The organism is actively proteolytic and forms sulphuretted hydrogen, reduces nitrates to nitrites, and ultimately to ammonia, and splits urea. It forms acid and gas from glucose and

does not ferment lactose, but its action on other sugars seems to vary; some strains ferment saccharose. Indol is produced by certain strains.

Bacillus pyocyaneus (*Pseudomonas æruginosa, Migula*).—This organism occurs in the form of minute rods 1·5 to 3 μ in length and less than 0·5 μ in thickness (Fig. 48). Occasionally two or three are found attached end to end. It is actively motile, possessing a terminal flagellum, and does not form spores. It stains readily with the ordinary basic stains, but is decolorised by Gram's method.

Cultivation.—It grows readily on all the ordinary media at the room temperature, the cultures being distinguished by the formation of a greenish pigment. On agar the growth forms an abundant slimy greyish layer which afterwards becomes greenish, and a bright green colour diffuses through the whole substance of the medium.

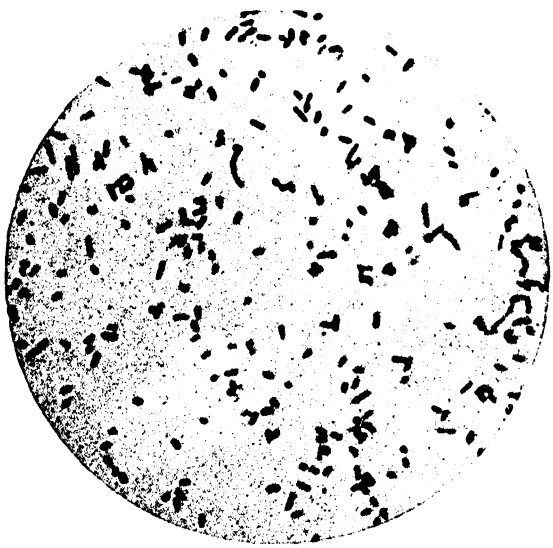


FIG. 48.—Bacillus pyocaneus; young culture on agar. Stained with weak carbol-fuchsin. ×1000.

In puncture cultures in peptone-gelatin a greyish line appears in twenty-four hours, and at its upper part a small cup of liquefaction forms within forty-eight hours. At this time a slightly greenish tint is seen in the superficial part of the gelatin. The liquefaction extends pretty rapidly, the fluid portion being turbid and showing masses of growth at its lower part. The green colour becomes more and more marked, and diffuses through the gelatin. Ultimately liquefaction reaches the wall of the tube. In plate cultures the colonies appear as minute whitish points, those on the surface being the larger. Under a low power of the microscope they have a brownish-yellow colour and show a nodulated surface, the superficial colonies being thinner and larger. Liquefaction soon occurs, the colonies on the surface forming shallow cups with small irregular masses of growth at the bottom, the deep colonies small spheres of liquefaction. Around the colonies a greenish tint appears. On potatoes the growth is an abundant reddish-brown layer resembling that of the glanders bacillus.

From the cultures there can be extracted by chloroform a coloured body, pyocyanin, which belongs to the aromatic series, and crystallises in the form of long, delicate bluish-green needles. On the addition of a weak acid its colour changes to red.

This organism has distinct pathogenic action in certain animals. Subcutaneous injection of small doses in rabbits may produce a local suppuration, but if the dose be large, spreading hæmorrhagic œdema results, which may be attended by septicæmia. Intraven-

ous injection may produce, according to the dose, rapid septicæmia with nephritis, or sometimes a more chronic condition of wasting attended by albuminuria.

Experimental Inoculation.—We shall consider chiefly the staphylococcus pyogenes aureus and the streptococcus pyogenes, as these have been most fully studied.

It may be stated at the outset that the occurrence of suppuration depends upon the number of organisms introduced into the tissues, the number necessary varying not only in different animals, but also in different parts of the same animal—a smaller number producing suppuration in the anterior chamber of the eye, for example, than in the peritoneum. The virulence of the organism also may vary, and corresponding results may be produced. Especially is this so in the case of the streptococcus pyogenes.

The *Staphylococcus aureus*, when injected subcutaneously in suitable numbers, produces an acute local inflammation, which is followed by suppuration, in the manner described above. If a large dose is injected, the cocci may enter the blood stream in sufficient numbers to cause secondary suppurative foci in internal organs. Intravenous injection in rabbits, for example, produces interesting results, which vary according to the quantity used. If a relatively small quantity be used, the cocci gradually disappear from the circulating blood; some become destroyed, while others settle in the capillary walls in various parts and produce minute abscesses. These are most common in the kidneys, where they occur both in the cortex and medulla as minute yellowish areas surrounded by a zone of intense congestion and hæmorrhage. Similar small abscesses may be produced in the heart wall, in the liver, under the periosteum and in the interior of bones, and occasionally in the striped muscles. Very rarely indeed, in experimental injection, do the cocci settle on the healthy valves of the heart. If, however, when the organisms are injected into the blood, there be any traumatism of a valve, or of any other part of the body, the show a special tendency to settle at these weakened points.

Experiments on the *human subject* have also proved the pyogenic properties of these organisms. Garré inoculated scratches near the root of his finger-nail with a pure culture, a small cutaneous pustule resulting; and by rubbing a culture over the skin of the forearm he caused a carbuncular condition which healed only after some weeks. Confirmatory experiments of this nature were made by Bockhart, Bumm, and others.

When tested experimentally, the staphylococcus pyogenes

albus has practically the same pathogenic effects as the staphylococcus aureus, though usually of less virulence ; it rarely gives rise to severe infection.

The *Streptococcus pyogenes* is an organism the virulence of which varies much according to the diseased condition from which it has been obtained, and also one which loses its virulence rapidly in cultures. Even highly virulent cultures, if grown under ordinary conditions, in the course of time lose practically all pathogenic power. By passage from animal to animal, however, the virulence may be much increased, and *pari passu* the effects of inoculation are correspondingly varied. Marmorek, for example, found that the virulence of a streptococcus can be enormously increased by growing it alternately (*a*) in a mixture of human blood serum and bouillon (*vide* p. 195), and (*b*) in the body of a rabbit ; ultimately, after several passages it possesses a super-virulent character, so that even an extremely minute dose introduced into the tissues of a rabbit produces acute septicæmia, with death in a few hours. It has been proved by Marmorek's experiments, and those of others, that the same species of streptococcus may produce at one time merely a passing local redness, at another a local suppuration, at another a spreading erysipelatous condition, or again a general septicæmic infection, according as its virulence is artificially increased. Such experiments are of extreme importance as explaining to some extent the great diversity of lesions in the human subject with which streptococci are associated.

Non-hæmolytic streptococci. — In animal experiments these types are usually of low virulence. After intravenous injection they show a great tendency to localise on the endocardium, producing endocarditis with vegetations. Some workers have found also a selective localisation in synovial membranes, the pericardium and in the cerebro-spinal fluid, their presence in the last being sometimes associated with choreiform movements. Such results have suggested the relationship of organisms of this type to acute rheumatism and its complications (*vide infra*). Non-hæmolytic streptococci isolated from the blood and urine in cases of nephritis have been found to reproduce in animals acute nephritis (G. Buchanan).

Bacillus coli.—The virulence of this organism also varies much, and can be increased by passage from animal to animal. Injection into the serous cavities of rabbits produces a fibrinous inflammation which becomes purulent if the animal lives sufficiently long. If, however, the virulence of the organism be of a high order, death takes place before suppuration is

established, and there is a septicæmic condition, the organisms occurring in large numbers in the blood. Intravenous injection of a few drops of a virulent bouillon culture usually produces a rapid septicæmia with scattered hæmorrhages in various organs.

Lesions in the Human Subject.—The following statement may be made with regard to the occurrence of the chief organisms mentioned, in the various suppurative and inflammatory conditions in the human subject. The account is, however, by no means exhaustive, as clinical bacteriology has shown that practically every part of the body may be the site of a lesion produced by the pyogenic bacteria.

It may also be noted that acute catarrhal conditions of mucous surfaces are in many cases to be ascribed to their presence.

The *staphylococci* are the most common causal agents in localised abscesses, in pustules on the skin, in carbuncles, boils, etc., in acute suppurative periostitis; they also occur frequently in catarrhs of mucous surfaces, in ulcerative endocarditis, and in various pyæmic conditions. They may also be present in cases of septicæmia.

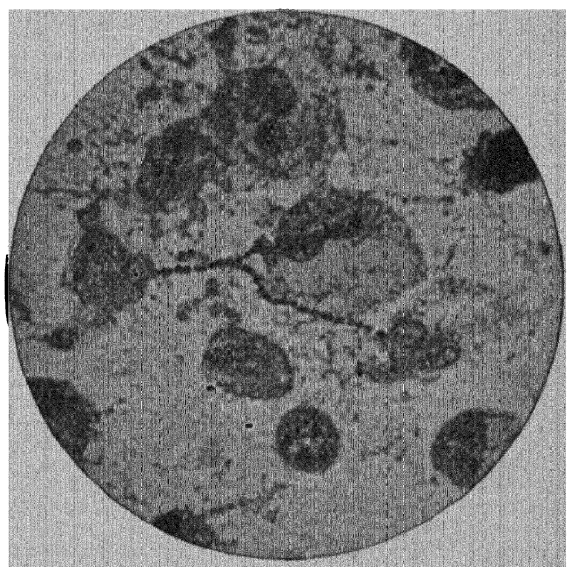


FIG. 49.—Streptococci in acute suppuration. Corrosive-fixed film; stained by Gram's method and safranin. ×1000.

Streptococci are especially found in spreading inflammation with or without suppuration; in diffuse phlegmonous and erysipelatous conditions, suppuration in serous membranes and in joints (Fig. 49). They are common in wound suppurations, usually along with other pyogenic organisms. During the war they were usually to be found in gun-shot wounds; at first the "enterococcus" abounded, at a later stage streptococcus pyogenes was the more common type. They also occur in acute suppurative periostitis and ulcerative endocarditis. Secondary abscesses in lymphatic glands and lymphangitis are also most frequently caused by streptococci. These lesions often occur when a virulent streptococcus has gained entrance through a prick or scratch in the skin, and in such cases there may be little or no inflammatory reaction at the site of entry of the infection.

They produce also fibrinous exudation on the mucous surfaces, leading to the formation of false membrane in many of the cases of non-diphtheritic inflammation of the throat, which are met with in scarlatina [1] and other conditions, and they are also the organisms most frequently present in acute catarrhal inflammations in this situation. Epidemics of sore throat, frequently of a severe type, have been attributed to streptococci spread by milk and derived from bovine mastitis. Both hæmolytic and non-hæmolytic streptococci are found in the broncho-pneumonic conditions occurring as complications of other infections, *e.g.* influenza. In puerperal sepsis they are frequently found in pure or mixed infection, and they appear to be the most frequent cause of puerperal septicæmia. In a certain proportion of cases they also produce peritonitis secondary to appendicitis. In pyæmia they are frequently present, in some cases associated with other pyogenic organisms. Some cases of enteritis in infants—streptococcic enteritis—are also apparently due to a streptococcus, which is usually of the " enterococcus " type.

Non-hæmolytic streptococci.—All are generally of lower virulence and associated with less acute lesions. They are slowly invasive and produce a low type of inflammation in which suppuration and tissue destruction are not marked. They are frequent in tonsillitis, otitis, dental abscess, pyorrhœa alveolaris, and bronchitis. They may occur, however, in broncho-pneumonia and even in septicæmic conditions, *e.g.* puerperal (*vide infra*). The condition of subacute infective endocarditis (*vide infra*) is due to this type of organism. Their association with acute rheumatism is referred to later.

The *Bacillus coli* is found in a great many inflammatory and suppurative conditions in connection with the alimentary tract —for example, in suppuration in the peritoneum, or in the extra-peritoneal tissue with or without perforation of the bowel, in the peritonitis following strangulation of the bowel, in appendicitis and the lesions following it, in suppuration in and around the bile ducts, etc. It may also occur in lesions in other parts of the body—endocarditis, pleurisy, etc., which in some cases are associated with lesions of the intestine, though in others such cannot be found. It is also frequently present in inflammation of the urinary passages, cystitis, pyelitis, abscesses in the kidneys, etc., these lesions being in fact most frequently caused by this group of organisms (Fig. 50).

[1] True diphtheria may also occasionally be associated with this disease, usually as a sequel.

In certain cases of enteritis it is possibly the causal agent, though this is difficult of proof, as it is much increased in numbers in practically all abnormal conditions of the intestine. We may remark that it has been repeatedly proved that the bacillus coli cultivated from various lesions is more virulent than the ordinary intestinal strains, its virulence having been heightened by growth in the tissues.

The *Micrococcus tetragenus* is often found in suppurations in the region of the mouth or in the neck, *e.g.* dental abscess, and also occurs in various lesions of the respiratory tract, in phthisical cavities, abscesses in the lungs, etc. Sometimes it is present alone, and probably has a pyogenic action in the human subject under certain conditions. In most cases it is associated with other organisms. During the war, cases of general infection along with pneumonic symptoms were recorded in soldiers, the organism having been isolated from the blood; recovery was the rule. Cases of pyæmia have been described in which this organism was found in a state of purity in the pus in various situations. In this latter condition the pus has been described as possessing an oily, viscous character, and as being often blood-stained.

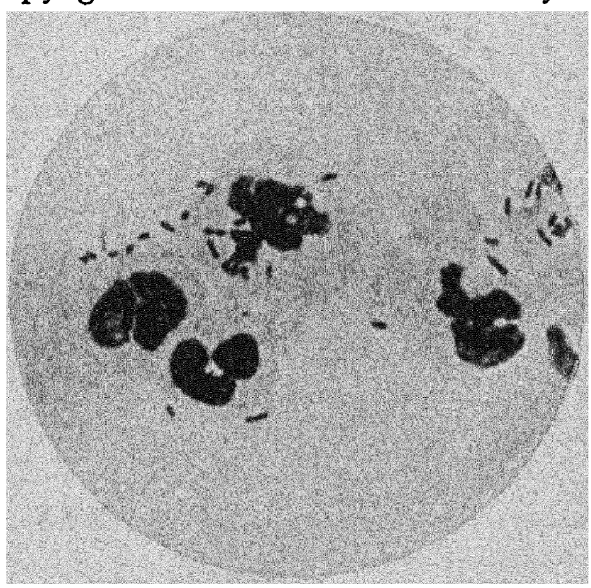


FIG. 50.—Film from urinary sediment showing B. coli. ×1000.

The *Bacillus pyocyaneus* (the organism of "green" or "blue" pus) is rarely found alone, though it is not infrequent along with other organisms. We have met with it several times in cases of multiple abscesses, in association with the staphylococcus pyogenes aureus. It is present along with other organisms in a large proportion of old suppurating war wounds. Cases of disease in children have been described in which the bacillus pyocyaneus has been found throughout the body; in these cases the chief symptoms have been fever, gastro-intestinal irritation, pustular or petechial eruptions in the skin, and general marasmus. It has also been said to be constantly present in pemphigus, and

it certainly occurs in some cases of this disease. It sometimes occurs in cystitis and pyelitis.

Bacillus proteus.—Although some cases of pure infection by this organism have been described, *e.g.* pleurisy, meningitis from middle-ear disease, etc., the bacilli generally occur along with other organisms in septic inflammations, such as cystitis and pyelitis, endometritis, peritonitis, etc. B. proteus is common also in gun-shot and other contaminated wounds.

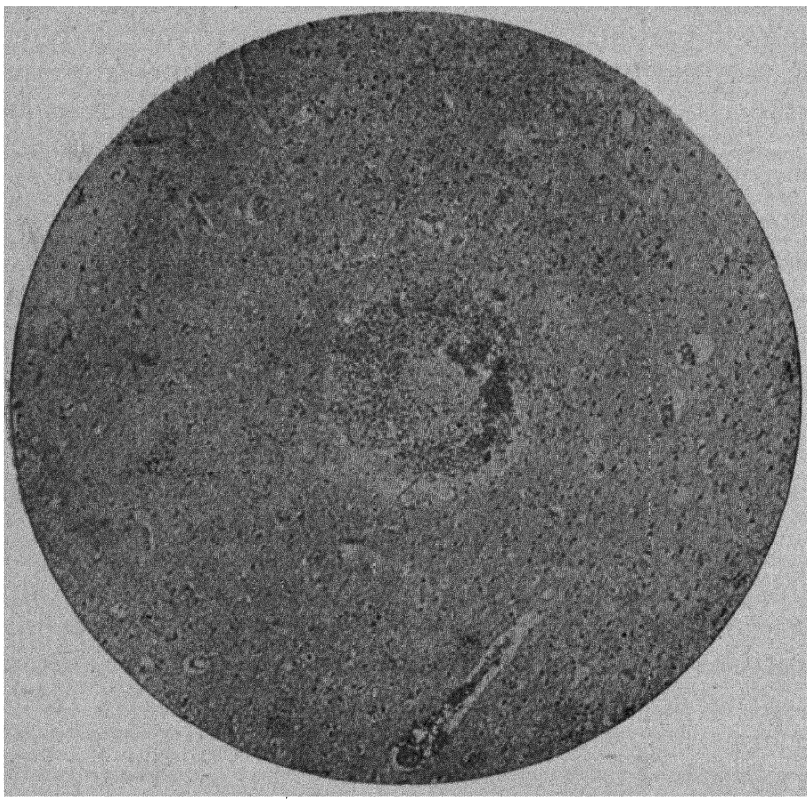


FIG. 51.—Minute focus of commencing suppuration in brain—case of acute ulcerative endocarditis. In the centre a small hæmorrhage; to right side dark masses of staphylococci; zone of leucocytes at periphery.
Alum-carmine and Gram's method. ×50.

Cases of severe gastro-enteritis apparently due to it have been described (Horowitz); and the organism is a frequent concomitant of B. dysenteriæ infection, occurring often in large numbers in the stools in late stages of the disease.

Inflammatory and suppurative conditions, associated with the organisms of special diseases, will be described in the respective chapters.

Mode of Entrance and Spread.—Many of the organisms described have a wide distribution in nature, and many also

are present on the skin and mucous membranes of healthy individuals. Staphylococci and non-hæmolytic streptococci are constant commensals in the mouth, nose, and throat. Even hæmolytic streptococci may occur normally in the throat, and particularly in the tonsillar crypts. The entrance of these organisms into the deeper tissues when a surface lesion occurs can be readily understood. Their action will, of course, be favoured

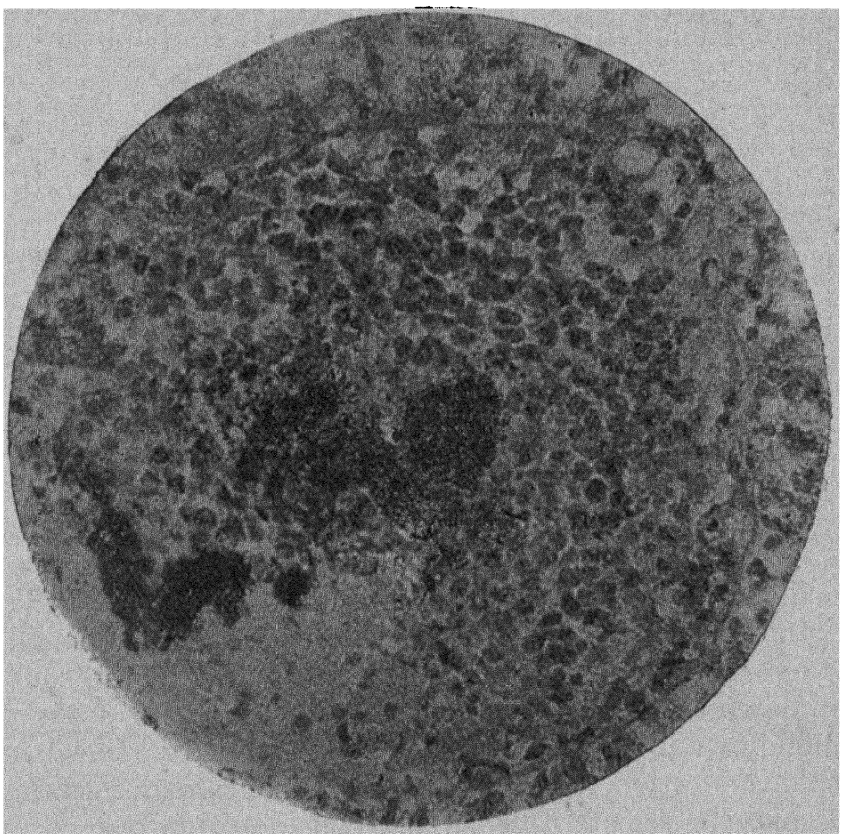


FIG. 52.—Secondary infection of a glomerulus of kidney by the staphylococcus aureus, in a case of ulcerative endocarditis. The cocci (stained darkly) are seen plugging the capillaries and also lying free. The glomerulus is much swollen, infiltrated by leucocytes, and partly necrosed.
Paraffin section; stained by Gram's method and Bismarck-brown. ×300.

by any condition of depressed vitality. Though in normal conditions the blood is bacterium-free, we must suppose that from time to time a certain number of such organisms gain entrance to it from trifling lesions of the skin or mucous surfaces, the possibilities of entrance from the latter being especially numerous. In most cases they are killed by the action of the healthy plasma or cells of the body, and no harm results. If, however, there be a local weakness, they may settle in that part

and produce suppuration, and from this other parts of the body may be infected. Such a supposition as this is necessary to explain many inflammatory and suppurative conditions met with clinically. In some cases of multiple suppurations due to staphylococcus infection, only an apparently unimportant surface lesion is present; whilst in others no lesion can be found to explain the origin of the infection. The term *cryptogenetic* has been applied by some writers to such cases in which the original point of infection cannot be found, but its use is scarcely necessary.

The *paths of secondary infection* may be conveniently summarised thus: *First*, by lymphatics; in this way the lymphatic glands may be infected, and also serous sacs in relation to the organs where the primary lesion exists. *Second*, by natural channels, such as the ureters and the bile ducts, the spread being generally associated with an inflammatory condition of the lining membrane. In this way the kidneys and liver respectively may be infected. *Third*, by the blood vessels: (*a*) by a few organisms gaining entrance to the blood from a local lesion, and settling in a favourable nidus or a damaged tissue, the original path of infection often being obscure; (*b*) by a septic phlebitis with suppurative softening of the thrombus and resulting embolism; and we may add (*c*), by a direct extension along a vein, producing a spreading thrombosis and suppuration within the vein. In this way suppuration may spread along the portal vein to the liver from a lesion in the alimentary canal, the condition being known as pylephlebitis suppurativa.

Although many of the lesions produced by the bacteria under consideration have already been mentioned, certain conditions may be selected for further consideration on account of their clinical importance or bacteriological interest.

Endocarditis.—There is now strong evidence that all cases of acute endocarditis are due to bacterial infection. In the simple or vegetative form, so often the result of acute rheumatism, the micrococcus rheumaticus (p. 258) has been cultivated from the valves in a certain number of cases, and is probably the causal agent in most instances. In this type the number of organisms in the lesions is small.

Endocarditis of the more severe or ulcerative type may be produced by various organisms, chiefly pyogenic. The most destructive types are produced by the hæmolytic streptococci and by staphylococci, the former being the commoner agents. The condition of *subacute infective endocarditis* has come to be recognised as almost a specific disease entity and is nearly

always due to non-hæmolytic streptococci. The condition is one of considerable duration and is almost invariably fatal. The left side of the heart is most frequently infected, and large chronic vegetations form with little or no tissue destruction. Embolism may result. There is marked splenic enlargement associated with the condition and frequently a glomerular nephritis.

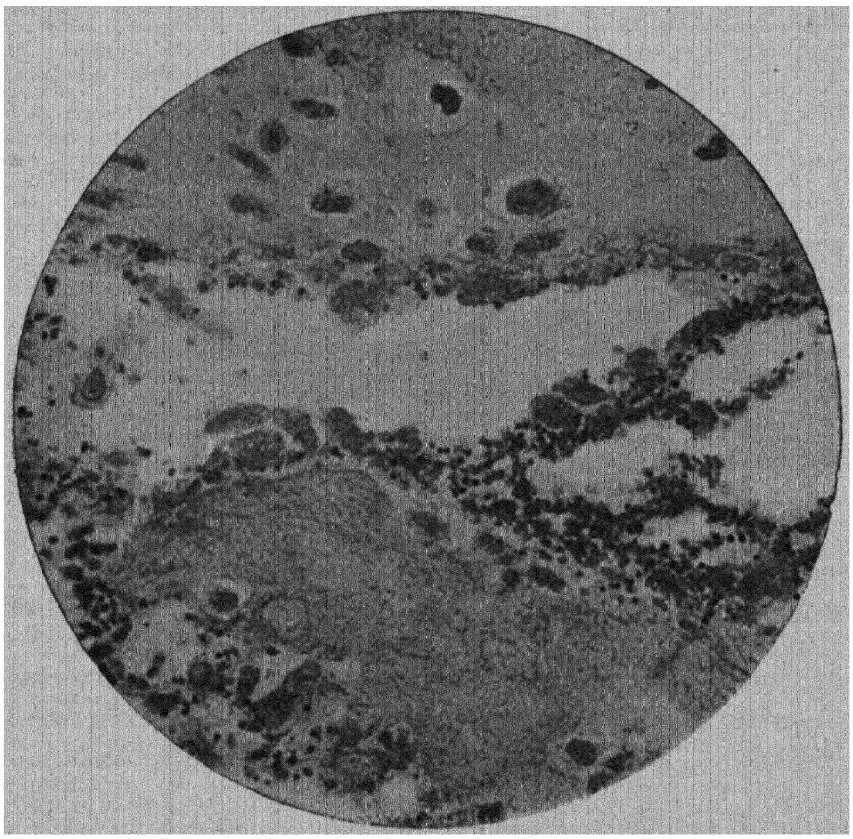


FIG. 53.—Section of a vegetation in ulcerative endocarditis, showing numerous staphylococci lying in the spaces. The lower portion is a fragment in process of separation.
Stained by Gram's method and Bismarck-brown. ×600.

Bacteriæmia is frequent but not invariable. The organisms are present in the vegetations.

This condition would appear to be intermediate between the simple endocarditis and the ulcerative types in which there is marked destruction of tissue. Thus in streptococcal endocarditis generally, all degrees of severity may be observed and the simple rheumatic endocarditis might be regarded as the mildest example. In some cases of ulcerative endocarditis following pneumonia the pneumococcus is present; in these the vegetations often reach

a large size and have not so much tendency to break down as in the case of staphylococcus infections. In some cases the bacillus coli has been found, and occasionally in endocarditis following typhoid the typhoid bacillus has been described as the organism present. The meningococcus and also the gonococcus have been shown to affect the heart valves (p. 306), though such occurrences are relatively rare. Tubercle nodules on the heart valves have been found in a few cases of acute tuberculosis, though no vegetative or ulcerative condition is usually produced.

Experimental.—Occasionally ulcerative endocarditis is produced by the simple intravenous injection of staphylococci or streptococci into the circulation, but this is a very rare occurence. It often follows, however, when the valves have been previously injured. Orth and Wyssokowitsch at a comparatively early date produced the condition by damaging the aortic cusps by a glass rod introduced through the carotid, and afterwards injecting staphylococci into the circulation. Similar experiments have since been repeated with streptococci, pneumococci, and other organisms, with like result. Ribbert found that if a potato culture of the staphylococcus aureus were rubbed down in salt solution so as to form an emulsion, and then injected into the circulation, some minute fragments became arrested at the attachment of the chordæ tendineæ and produced an ulcerative endocarditis.

Acute Suppurative Periostitis and Osteomyelitis.—Special mention is made of this condition on account of its comparative frequency and gravity. The great majority of cases are caused by the pyogenic cocci, of which one or two varieties may be present, the staphylococcus aureus, however, occurring most frequently. Pneumococci have been found alone in some cases, and in a considerable number of cases following typhoid fever the bacillus typhosus has been found alone. In others, again, the bacillus coli is present.

The affection of the periosteum or interior of the bones by these organisms, which is especially common in young subjects, may take place in the course of other affections produced by the same organisms or in the course of infective fevers, but in a great many cases the path of entrance cannot be determined. In the course of this disease serious secondary infections are always very liable to follow, such as small abscesses in the kidneys, heart wall, lungs, liver, etc., suppurations in serous cavities, and ulcerative endocarditis ; in fact, some cases present the most typical examples of extreme general staphylococcus infection. The entrance of the organisms into the blood stream from the lesion of the bone is especially favoured by the arrangement of the veins in the bone and marrow.

Experimental.—Multiple abscesses in the bones and under the periosteum may follow simple intravenous injection of the pyogenic cocci into the blood, and are especially liable to be formed when young animals are used. These abscesses are of small size, and do not spread in the same way as in the natural disease in the human subject.

In experiments on healthy animals, however, the conditions are not analogous to those of the natural disease. We must presume that in the latter there is some local weakness or susceptibility, which enables the few organisms which have reached the part by the blood to settle and multiply. Moreover, if a bone be experimentally injured, *e.g.* by actual fracture or by stripping off the periosteum before the organisms are injected, then a much more extensive suppuration occurs at the injured part.

Erysipelas.—A spreading inflammatory condition of the skin may be produced by a variety of organisms, but the disease in the human subject in its characteristic form is almost invariably due to a streptococcus, as was shown by Fehleisen in 1884. He obtained pure cultures of the organism, and gave it the name of Streptococcus erysipelatis; and, further, by inoculations in the human subject as a therapeutic measure in malignant disease, he was able to reproduce erysipelas. As stated above, however, one after another of the supposed points of difference between the streptococcus of erysipelas and the streptococcus pyogenes of suppuration has broken down. It must be noted, however, that erysipelas passes from patient to patient as erysipelas, and purulent conditions due to streptococci do not appear liable to be followed by erysipelas. On the other hand, the connection between erysipelas and puerperal septicæmia is well established clinically.

In a case of erysipelas the streptococci are found in large numbers in the lymphatics of the cutis and underlying tissues, just beyond the swollen margin of the inflammatory area. As the inflammation advances they gradually die out, and after a time their extension at the periphery comes to an end. The streptococci may extend to serous and synovial cavities and set up inflammatory or suppurative change—peritonitis, meningitis, and synovitis may thus be produced.

Puerperal Sepsis.—While other pyogenic organisms are frequently associated with puerperal sepsis, *e.g.* staphylococci or B. coli, the streptococci represent the most prevalent and probably the most formidable type of organisms responsible for such infection. Though only localised inflammatory and suppurative lesions may result, general septicæmia is not infrequent, and then the infection is generalised almost from the outset and presents a clinical and pathological picture of extreme

virulence. Puerperal sepsis is regarded as essentially due to an exogenous infection with virulent strains or the result of infection from the external parts, but it must be admitted that it is not infrequently endogenous, the organisms entering the uterus or being introduced mechanically from the lower genital passages. It has been shown that non-hæmolytic and even hæmolytic streptococci occur in the vagina; FitzGibbon and Bigger have stated that non-hæmolytic streptococci are found in the vagina in two of every three pregnant women. Both hæmolytic and non-hæmolytic streptococci occur in puerperal septicæmia, the latter even in fatal cases. The question of the factors concerned in predisposing to such infections remains undecided and constitutes an important problem in obstetric practice.

Acute Rheumatism.—There are many facts which point to the infective nature of this disease, and investigations from this point of view have yielded important results. Of the organisms isolated, the one which appears to have strongest claims is a small coccus observed by Triboulet, and by Westphal and Wassermann, the characters and action of which were first investigated in this country by Poynton and Paine. It is now usually known as the *Micrococcus rheumaticus*. The organism is sometimes spoken of as a diplococcus, but it is best described as a Gram-positive streptococcus growing in short chains; in the tissues, however, it usually occurs in pairs. Like other streptococci it ferments various sugars with acid production, and usually clots milk after incubation for two days; on blood agar it alters the hæmoglobin to a brownish colour. Its growth on media generally is more luxuriant than that of the streptococcus pyogenes, and it grows well on gelatin at 20° C. Injection of pure cultures in rabbits often produces polyarthritis and synovitis, valvulitis and pericarditis, without any suppurative change—lesions which are not produced by the ordinary streptococci (Beattie). In one or two instances choreiform movements have been observed after injection. The organism is most easily obtained from the substance of inflamed synovial membrane where it is invading the tissues; a part where there is special congestion should be selected as being most likely to give positive results. It is only occasionally to be obtained from the fluid in joints. It has also been cultivated from the blood in rheumatic fever, from the vegetations on the heart valves, and from other acute lesions; in many cases, however, cultures from the blood give negative results. Beattie has shown that in rabbits the experimental arthritis is attended by the main features of acute or subacute rheumatism in man, namely,

the rapidity with which the affection passes from joint to joint, the tendency to relapses, the contributory effects of exposure to cold, and the absence of gross anatomical changes in the joints. Poynton and Paine cultivated the streptococcus from the cerebro-spinal fluid in three cases where chorea was present, and also detected it in the membranes of the brain. They consider that this disease is probably of the nature of a slight meningo-myelitis produced by the organism. The facts already accumulated speak strongly in favour of this organism being causally related to rheumatic fever, though this cannot be considered completely proved. Andrewes finds that the organism has the same cultural characters and fermentative effects as the streptococcus fæcalis, a common inhabitant of the intestine. Even, however, if the two organisms were the same, it might well be possible that rheumatic fever is due to an infection of the tissues by this variety of streptococcus. The clinical data, in fact, rather point to rheumatic fever being due to an infection by some organism frequently present in the body, brought about by some state of predisposition or acquired susceptibility. In view of the results recorded in some cases of "enterococcus" infection with myalgia, etc., the question is again raised as to the identity of the organisms. Beattie and Yates have brought forward important evidence to show that the joints do not become infected *post mortem* with the streptococci of the alimentary canal as a terminal phenomenon, and that accordingly the finding of the micrococcus rheumaticus in the joints has an important etiological significance.

In this connection it is to be noted that the lesions produced in animals by inoculation with this organism may also result from the intravenous injection of other non-hæmolytic streptococci, *e.g.* those isolated from the mouth and throat. This is particularly true in regard to the endocardial lesions and the relationship of streptococci to endocarditis has been referred to above. The etiology of acute rheumatism, which is of the nature of a specific infective disease, still remains a matter of uncertainty, and the data in support of the streptococcal origin of the disease are not sufficiently uniform or convincing.

Conjunctivitis.—A considerable number of organisms are concerned in the production of conjunctivitis and its associated lesions. Of these, certain appear to be specially associated with this region. Thus a small organism, generally known as the Koch-Weeks bacillus (*Hæmophilus conjunctivitidis*), is the most common cause of acute contagious conjunctivitis, especially prevalent in Egypt, but also common in this country. This

organism is very minute, being little more than 1 μ in length, Gram-negative, and morphologically resembles the influenza bacillus ; its conditions of growth are less restricted, as it grows well on serum-agar without blood. On this medium it produces minute transparent colonies like drops of dew. The obtaining of pure cultures is a matter of considerable difficulty, and it is nearly always accompanied by the xerosis bacillus. It can readily be found in the muco-purulent secretion by staining films with weak (1 : 10) carbol-fuchsin, and is often to be seen in the interior of leucocytes (Fig. 54). The organism described in the literature as " Müller's bacillus " is apparently a closely allied type. It was cultivated by him in a considerable proportion of cases of trachoma, but its relation to this condition is doubtful.

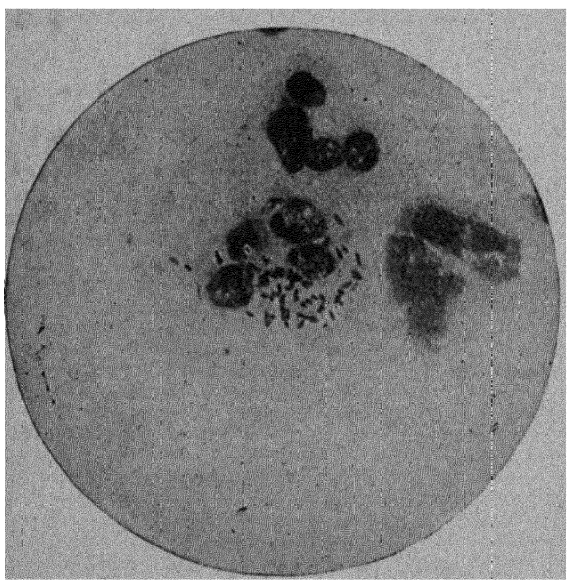


FIG. 54.—Film preparation from a case of acute conjunctivitis, showing Koch-Weeks bacilli, chiefly contained within a leucocyte. (From a preparation by Dr. Inglis Pollock.) ×1000.

Another bacillus which is now well recognised is the diplo-bacillus of conjunctivitis first described by Morax ; it is often known as the Morax-Axenfeld bacillus (*Hæmophilus lacunatus*). It is especially common in the more subacute cases of conjunctivitis. Eyre found it in 2·5 per cent. of all cases of conjunctivitis. This organism is a small plump bacillus, measuring 1×2 μ and usually occurring in pairs, or in short chains of pairs (Fig. 56). It is non-motile, does not form spores, and is decolorised by Gram's method. It does not grow on the ordinary gelatin and agar media, the addition of blood or serum being necessary. On solidified serum it forms small rounded colonies which produce small pits of liquefaction ; hence it has been called the *Bacillus lacunatus*. In cultures it is distinctly pleomorphous, and involution forms also occur. It is non-pathogenic to the lower animals. The xerosis bacillus, which is a small diphtheroid organism (Fig. 127), has been found in xerosis of the conjunctiva, in follicular conjunctivitis, and in other conditions ; it appears to occur

frequently also in the normal conjunctiva. It is doubtful whether it has any pathogenic action of importance. Acute

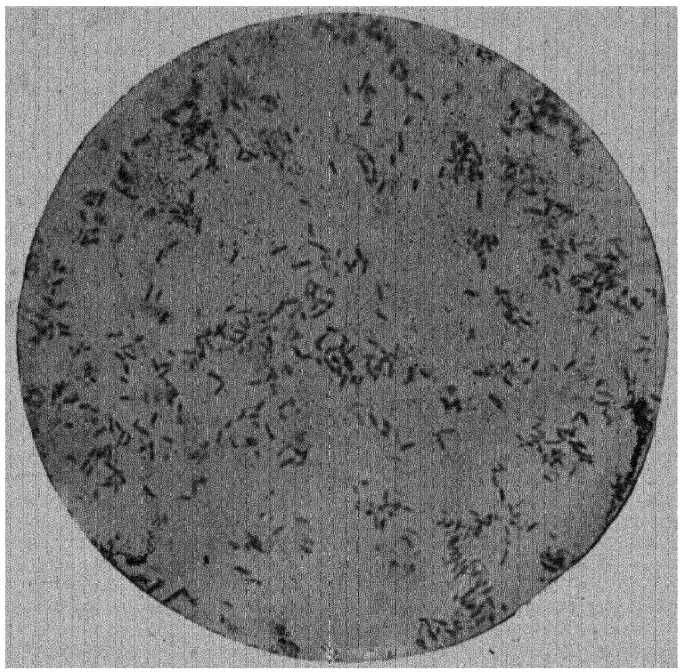


FIG. 55.—Koch-Weeks bacillus, from a young culture on blood agar. Stained with weak carbol-fuchsin. ×1000.

conjunctivitis is also produced by the pneumococcus, epidemics of the disease being sometimes due to this organism, and also to streptococci and staphylococci; the staph. albus is, however, found in the conjunctival sac, often in large numbers, as a normal commensal. True diphtheria of the conjunctiva caused by the B. diphtheriæ also occurs, whilst in gonorrhœal conjunctivitis, often of an acute purulent type, the gonococcus is present (p. 305).

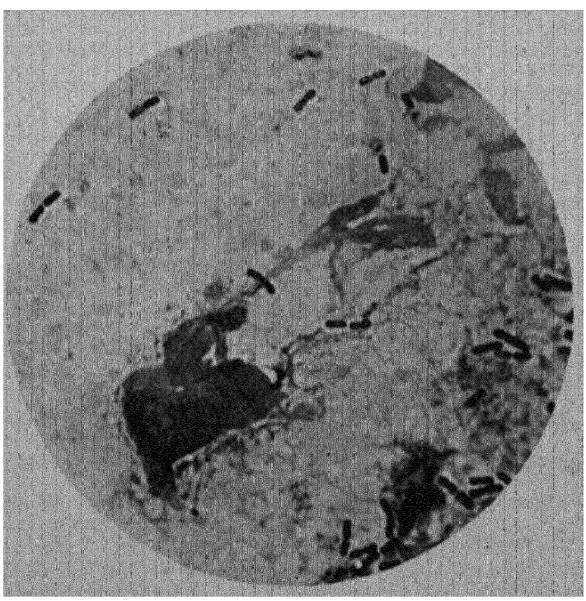


FIG. 56.—Film preparation of conjunctival secretion, showing the Morax diplo-bacillus of conjunctivitis. ×1000.

Acne.—In the pus of acne lesions and also in the comedones a bacillus, now generally known as the *acne bacillus* (*Corynebacterium acnes*), may be found in large numbers. The organism was first described by Unna and afterwards cultivated by Sabouraud. It occurs in the form of short rods, sometimes swollen at one end, and measuring about 2 μ in length and rather less than 0·5 μ in thickness. It often shows a beaded appearance like that of a diphtheroid bacillus. It stains readily with the basic aniline dyes and retains the stain in Gram's method. It grows best in the presence of only a small quantity of atmospheric oxygen, being a micro-aerophilic organism. In shake or stab cultures, growth is at a maximum in a limited zone about half an inch from the surface. It can be cultivated by the usual anaerobic methods, but no growth occurs as a rule under aerobic conditions. Glucose agar is a suitable medium. In such a medium after three or four days' incubation at 37° C. small whitish colonies appear, which when examined under a low magnification are seen to have a lenticulate shape. The organism shows considerable pleomorphism—coccoid, diphtheroid, and filamentous types being present, as well as irregular bizarre forms. Some observers have also obtained surface growth on ordinary agar, especially after the organism has been cultivated for some time under anaerobic conditions. Its relation to the suppuration in acne has been a matter of dispute, some holding that it is the cause of the suppuration, whilst others maintain that this is due to pyogenic cocci.

Scarlatina.—It has been long recognised that streptococci are usually present in large numbers in the throat in scarlatina, and that many of the complications of this disease are undoubtedly streptococcal. At one time, streptococci were regarded, in virtue of their occurrence in the disease, as the likely etiological agent, and the associated strains were designated "Streptococcus scarlatinæ." Thus, in 1887, Klein, as a result of the investigation of milk-borne outbreaks, claimed that the disease was due to a definite type of streptococcus, but subsequent work showed that the scarlatina streptococcus was similar in its cultural characters to the pyogenic strains and had no specific pathogenic effects on animals. Later views tended to reverse the original idea, although the almost constant occurrence of hæmolytic streptococci in the throat was well recognised, and it was supposed that these organisms represented a secondary infection and that the primary etiological agent was a different and undetermined type of micro-organism possibly belonging to the category of the filterable viruses. Attempts to demonstrate a

filterable virus of scarlatina, however, led to negative or inconclusive results.

In 1923, George and Gladys Dick were able to produce scarlet fever in a volunteer by infecting the throat with a culture of a hæmolytic streptococcus isolated from a known case of the disease, and in 1924 they demonstrated that the scarlatina streptococcus produces a diffusible " toxin," present in filtrates from cultures, which, injected intracutaneously in persons susceptible to the disease, elicits a reaction of cutaneous erythema and inflammation ; while non-susceptibles (*e.g.* convalescents) fail to show the reaction. This reaction is therefore analogous to the Schick reaction for susceptibility to diphtheria (*q.v.*), and is now designated the Dick reaction. They also produced symptoms of scarlet fever in volunteers by injecting the filtrate of a culture of the scarlatina streptococcus. The validity of the Dick reaction has been well established by Zingher and others. Thus persons during the early stage of the disease (first three days), in the great majority of cases, exhibit a markedly positive reaction illustrating apparently their susceptibility to the " toxin " and the absence of any natural or acquired immunity. On the other hand, convalescents usually show a negative reaction which suggests the acquisition of an antitoxic immunity as a result of the infection. It has also been shown by the so-called Schultz-Charlton reaction that the serum of convalescents contains neutralising substances, *i.e.* the intracutaneous injection of the convalescent serum produces a local blanching or " extinction " of the rash in an active case. Further, the serum of convalescents neutralises the Dick toxin when mixed with it and injected into the skin of a known susceptible. These facts, together with the constant occurrence of hæmolytic streptococci in the throat, strongly support the view that scarlatina is due to infection of the throat with a hæmolytic streptococcus capable of producing a specific diffusible toxin, which is responsible for the general manifestation of the disease, and that the infection is in the first instance local, the general condition being essentially a toxæmia.

Various workers, particularly Bliss, Tunnicliff, Gordon, Stevens and Dochez, and Eagles, have claimed that most of the scarlet fever strains fall into one serological group and differ serologically from other hæmolytic streptococci. It seems doubtful, however, whether one single type of streptococcus is responsible for scarlet fever. It has been shown that hæmolytic streptococci from cases of erysipelas, infected wounds and normal throats, may produce toxins similar to that obtained

from known scarlet fever strains. Park, Williams, and Krumwiede have reported that of sixty scarlet fever strains, only 28 per cent. belong to one serological group, and that 15 per cent. of strains from non-scarlet cases also belong to this group. According to Smith, the majority of scarlatina strains studied by him can be classified into two serological types. It seems probable that the antigenic characters of the streptococci associated with scarlatina are more varied than has been supposed, and it is doubtful whether these organisms can be definitely separated into one or more serological groups distinct from other hæmolytic streptococci. Further investigations are required on this subject.

An obstacle to the experimental study of the etiology of the disease is the fact that no laboratory animal is susceptible to the toxin. The only certain criterion, therefore, of the identity of a scarlatina-producing streptococcus depends on whether culture filtrates in suitable dilution yield a positive Dick reaction in known susceptibles (*e.g.* early scarlet fever cases) and negative reactions in known negative reactors (*e.g.* convalescents). The neutralisation by an antitoxic serum (*vide infra*) of the reacting property of the filtrate would constitute a further test.

The logical outcome of recent work on scarlet fever has been (1) the active immunisation with toxin for the prophylaxis of the disease as in active immunisation against diphtheria (*q.v.*), and (2) the treatment of the disease by passive immunisation with antitoxic sera. It has been shown that by immunisation of horses with the toxin, a neutralising antiserum can be obtained. and such sera are now being utilised in treatment with promising results. It is still too early, however, to draw any definite conclusions as to the actual value of these applications of recent knowledge regarding the etiology of scarlet fever.

The Dick reaction has elicited the ages of maximum susceptibility to scarlet, viz. between six months and three years. Over twenty years of age about 80 per cent. of persons are immune.

While the diffusible principle of the S. scarlatinæ, which is now spoken of as " toxin," has been commonly regarded as analogous to other bacterial exotoxins, it presents certain striking differences. Thus it possesses an unusually high degree of thermostability, being only destroyed at 100° C. No toxic effects have been demonstrated in animals by injection of this substance. It has therefore been suggested that the scarlet fever rash is a phenomenon of hypersensitiveness to the proteins of the streptococcus, and that the Dick reaction is an index of sensitiveness (*vide* Chapter VI.). Such supersensitiveness is supposed to be the

result of pre-existing subinfection. Guinea-pigs which normally fail to react to the Dick toxin can apparently be sensitised by injection of "toxin" or cultures so that a subsequent intracutaneous injection produces a reaction similar to the Dick reaction. It is, however, doubtful whether this reaction is strictly analogous with the Dick reaction in the human subject.

The question therefore remains unsettled as to whether the toxic manifestations of scarlet fever are due to an exotoxin similar to other bacterial exotoxins or to a reaction of hypersensitiveness to the proteins of the S. scarlatinæ. The recent work has, however, clearly demonstrated the important relationship of hæmolytic streptococci to the disease, and throws light on the genesis of a disease for long regarded as etiologically obscure. An explanation is also probably afforded of the occurrence of the so-called "Surgical Scarlet Fever," the result of a streptococcal wound infection.

The Dick Reaction.—*Preparation of toxin.*—A suitable strain of streptococcus scarlatinæ is grown at 37° C. for five days in broth (P_H 7·5), containing 5 per cent. sterile defibrinated rabbit's blood. The culture is centrifuged at high speed, and the supernatant fluid decanted and filtered through a tested Berkefeld filter at low pressure. The filtrate, which is deeply hæmoglobin-tinted as a result of the hæmolysis in the culture, constitutes the "toxin." 0·5 per cent. phenol should be added for preservation. In the test a suitable dilution is used, *e.g.* 1 : 1000 in normal saline. Where a previously untested strain is used for the preparation of the toxin, the optimum dilution should be ascertained by tests in early and convalescent scarlet cases (*vide supra*). The amount injected intracutaneously is from 0·1 to 0·2 c.c. The injection is usually made on the forearm. A control test should be carried out simultaneously, using the same amount of toxin after heating at 100° C. for one hour. The toxin may be kept in a 1 : 100 dilution, and before carrying out tests the necessary amount of the final dilution can be prepared from the stock. An equal volume of it is also inactivated by heating for the control tests.

The technique of the injection is that used for other intradermal tests.

The positive reaction develops in six to twelve hours, and appears as a bright scarlet erythematous reaction often 30 mm. in diameter. Its maximum is usually attained within twenty-four hours, and it then gradually fades and has practically disappeared by the third day. Readings should be made not later than twenty-four hours. Usually the control test leads to

no definite reaction, but occasionally pseudo-reactions of varying degree are met with and are more persistent than the true reaction.

Mackie and M'Lachlan have utilised a simple method of obtaining a purified preparation of toxin in which filtration is omitted. The culture, after incubation for five days (when the added blood is almost completely lysed), is heated at 57° C. for one hour. This sterilises the culture and leads to a copious brownish precipitate which is removed by centrifuging. The supernatant fluid is then treated with 6 volumes of absolute alcohol and the resulting precipitate is centrifuged and finally redissolved in saline to the original volume of the culture. From this a 1 : 1000 (or other appropriate dilution) is made as in dealing with the filtrate preparation. This purified preparation, which eliminates much of the inactive material derived from the added blood, yields results identical with those of the ordinary preparations.

Purification of toxin preparations by precipitation with alcohol and re-solution in saline has been used also by Henry and Lewis.

Active Immunisation.—Zingher recommends for immunisation, weekly subcutaneous injections of a toxin dilution in saline made up so that 1 c.c. is equivalent to 500 "skin-test" doses. Three doses are given: (1) 0·2 c.c., (2) 0·5 c.c., and (3) 0·5 c.c. for children under twelve years, 1 c.c. for children over twelve, and 2 c.c. in the case of adults. A general rash with fever has been recorded in some cases after the initial dose. This treatment renders the previously positive Dick reaction either negative or less markedly positive.

The actual prophylactic value of such immunisation still remains to be decided by future experience.

Vaccination Treatment of Infections by the Pyogenic Cocci.—From his study of the part played by phagocytosis in the successful combat of the pyogenic bacteria by the body, Wright was led to advocate the treatment of such infections during their course by active immunisation by means of dead cultures of the infecting agent (for methods of preparation, see p. 139). The treatment is applicable when the infection is practically local, as in acne pustules, in boils, etc., but has also been applied in more acute conditions. (For the theoretical questions raised, see Immunity.) For an isolated furuncle, Wright recommended a dose of 50 to 100 millions staphylococci to be followed three or four days later by the injection of 250 to 300 millions, and for an incipient streptococcic lymphangitis a dose of 500,000 to 2,000,000 streptococci. In chronic staphylococcal infections the number of bacteria used for an injection is from 250,000,000 to 500,000,000, but a smaller number may give a good result, and the general principle to be adopted is to use the smallest dose necessary for a therapeutic effect. If it

is not practicable to use the strain derived from the lesion for the preparation of an " autogenous " vaccine, then laboratory cultures or the stock vaccines which are now in the market may be used ; in such cases it is well to use a " polyvalent " vaccine made from a mixture of strains ; in skin infections a mixture of staphylococcus aureus and albus may be employed. The treatment of various staphylococcus infections, such as pustular acne, boils, and chronic abscesses, by vaccines, has been carried out very extensively, in many cases with good result, and a similar statement is true of some streptococcic infections. Vaccine therapy has also been used in inflammatory and suppurative conditions due to other organisms, for example, infections of the genito-urinary tract with B. coli, where an autogenous vaccine with initial doses of from 10,000,000 to 50,000,000 may be employed ; gonococcal arthritis, where the initial dose is from 1,000,000 to 5,000,000 organisms ; chronic respiratory catarrh The case of the last can usually only be met by mixed vaccines on account of the presence of different species of bacteria, several of which may be potentially pathogenic ; in these circumstances the use of a mixed vaccine is purely empirical.

The treatment has also been applied in acute infections, *e.g.* with the pyogenic cocci, B. coli, etc., very small doses—*e g.* from 200,000 to 5,000,000—being given, but the method has not been attended by striking success. It is stated that better results have been obtained by the use of sensitised vaccines (*q.v.*), very small doses being here again employed.

Methods of Examination in Inflammatory and Suppurative Conditions.—These are usually of a comparatively simple nature, and include (1) microscopic examination, (2) the making of cultures.

(1) The pus or other fluids should be examined microscopically, first of all by means of film preparations in order to determine the characters of the organisms present. The films should be stained (*a*) by one of the ordinary solutions, such as carbol-thionin-blue (p. 105), or a saturated watery solution of methylene-blue ; and (*b*) by Gram's method. The use of the latter is of course of great importance as an aid in the recognition.

(2) The cultivation and separation of the organisms from the lesions are best attained by the method of successive strokes on agar plates or on agar slopes, the former being preferable (p. 73). In the case of an organism requiring a special medium, this of course is to be used. In the routine examination of streptococcal infections blood-agar may be used with advantage to distinguish the hæmolytic and non-hæmolytic types. Inoculation experiments may be carried out as occasion arises.

In cases of suspected blood infection the examination of the blood is to be carried out by the methods already described (p. 145). Reference is made on p. 148 to the methods of obtaining suitable specimens for bacteriological examination.

CHAPTER VIII

INFLAMMATORY AND SUPPURATIVE CONDITIONS, *CONTINUED*: THE ACUTE PNEUMONIAS, EPIDEMIC CEREBRO-SPINAL MENINGITIS

Pneumonia

Introductory.—Inflammatory changes in the lungs are the results of infection in different ways, and lead to a variety of structural changes. Thus different forms—lobar pneumonia, broncho-pneumonia, hypostatic pneumonia, and embolic pneumonia—are recognised. Acute lobar pneumonia, however, stands out in certain respects from the other forms and presents characters of high scientific interest. Its striking clinical features, and especially the course of the temperature, have long been recognised as resembling those of an acute specific fever. And while it was generally supposed to be caused by exposure to cold, such an explanation was by many considered unsatisfactory. Further, its occurrence in epidemics and the absence of history of exposure in many cases, supported the view that it was of the nature of a specific infection. Our knowledge of its etiology has been gradually evolved, rather than established by one discovery as has been the case with other infections; and years elapsed after the discovery of the pneumococcus before it gained general acceptance as the causal organism. This is due to several circumstances, but chiefly to the fact that the organism was found both in other lesions and in normal throats, and also to the difficulty experienced in producing a typical lobar pneumonia in animals. We shall, in the first place, deal with the characters of the pneumococcus and its relation to lobar pneumonia and afterwards consider the causation of other types of pneumonia.

Historical.—The first important work on the etiology of pneumonia was that of Friedländer (1882–83), who observed in the lungs capsulated cocci, which he isolated and showed to possess pathogenic properties. The situation was complicated by the subsequent observation that the injection into animals of the sputum of healthy

individuals frequently originated a septicæmic condition with the presence of capsulated cocci in the blood. The significance of the occurrence of this "sputum septicæmia" could not at that period be properly realised, as it was not recognised that an organism could produce different results in different animals, and therefore it was thought that the organisms described by Friedländer were not specifically related to pneumonia. Somewhat later, A. Fraenkel described diplococci in pneumonia which differed culturally from those of Friedländer. The work of Weichselbaum in 1886 elucidated the subject further. This observer, investigating 129 cases of various types of pneumonia, isolated, first and most frequently, an organism which he called the *Diplococcus pneumoniæ* (with a variant named by him the *Streptococcus pneumoniæ*), and which corresponded with Fraenkel's organism; second, an organism which he described as the *Bacillus pneumoniæ*, occurring less frequently and corresponding with that originally noted by Friedländer.

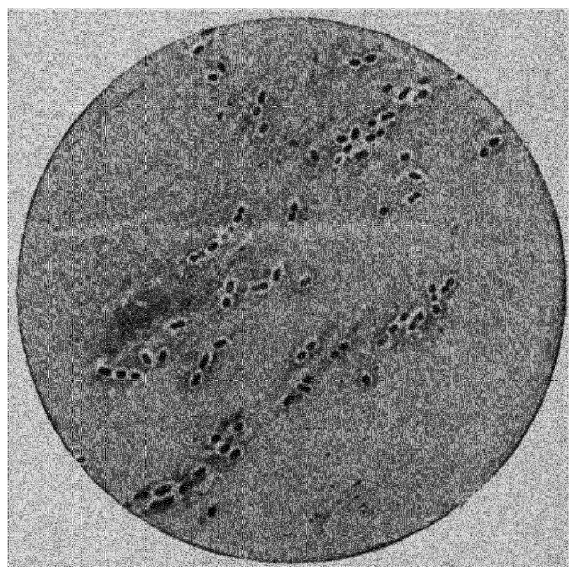


FIG. 57.—Film preparations of pneumonic sputum, showing numerous pneumococci (Fraenkel's) with unstained capsules; some are arranged in short chains. See also Plate I., Fig. 2.
Stained with carbol-fuchsin. ×1000.

The general result of all observations on pneumonia has been to establish that the organism described by Fraenkel, and now known as the *Pneumococcus*, is that of most frequent occurrence; it is the sole organism present in about 95 per cent. of cases of lobar pneumonia.

Pneumococcus (*Diplococcus pneumoniæ*): *Microscopic Characters.*—As seen in pneumonic sputum or exudate, the pneumococcus occurs in the form of a small oval coccus, about 1 μ in longest diameter, arranged generally in pairs (diplococci), but also in chains of four to ten (Fig. 57). The free ends are often pointed like a lancet, hence the name *Diplococcus lanceolatus* has also been applied to it. These cocci, in their typical form, have round them a capsule, which, in films stained by ordinary methods, usually appears as an unstained halo, but is sometimes stained more deeply than the ground of the preparation. This difference in staining depends, in part at least, on the amount of decolorisation to which the preparation has been subjected. The capsule is rather broader than the body of the coccus, and

has a sharply defined external margin ; it may be stained by the special methods already described. In sputum preparations the capsule of the pneumococcus may not be recognisable, and the same is sometimes true of preparations of pneumococcal exudates in the lung or in other parts of the body. The organism takes up the basic aniline stains with great readiness and is Gram-positive. In any lesion many degenerated individuals may occur, and these have often become Gram-negative.

Cultivation.—It is often difficult, and sometimes impossible, to isolate this coccus in culture directly from pneumonic sputum. On culture media it has not a vigorous growth, and when mixed with other bacteria it is apt to be overgrown by the latter. To get a pure culture it is best to insert a small piece of the sputum beneath the skin of a rabbit or a mouse. In about twenty-four to forty-eight hours the animal will die, with numerous capsulated pneumococci throughout its blood. From the heart-blood cultures can be easily obtained. Cultures can also be got *post mortem* from the lungs of pneumonic patients by streaking agar or blood agar with a scraping taken from the area of acute congestion or commencing red hepatisation, and incubating at 37° C. This method is also sometimes successful in the case of sputum.

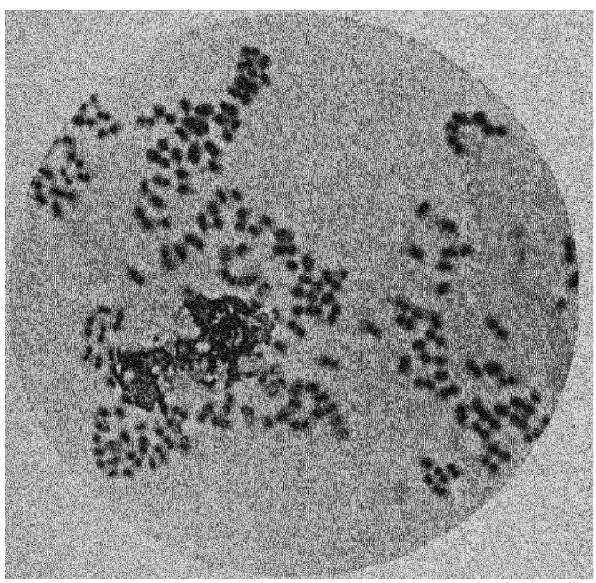


FIG. 58.—Fraenkel's pneumococcus in serous exudation at site of inoculation in a rabbit, showing capsules.
Stained by Rd. Muir's method. ×1000.

The appearances presented in cultures by different varieties of the pneumococcus vary somewhat. It grows best on blood serum, on Pfeiffer's blood agar, or on boiled blood agar. It often grows well on ordinary agar or in bouillon, but not so well on glycerin agar. In a stroke culture on *blood serum* growth appears as an almost transparent pellicle along the track, with isolated colonies at the margin. On *agar* media it is more manifest, but otherwise has similar characters. On agar, colonies are very transparent, but under a low power of the microscope appear

to have a compact finely granular centre and a pale transparent periphery; after forty-eight hours they increase slightly in size and present a depressed centre (Fig. 59). The appearances are similar to those of a culture of streptococcus pyogenes, but the growth is less vigorous, and is more delicate in appearance. A similar statement also applies to cultures in *gelatin* at 22° C., growth in a stab culture appearing as a row of minute points which remain of small size; there is no liquefaction of the medium. Often, however, no growth occurs in gelatin. In *bouillon* (which must be made from fresh meat—rabbit muscle



FIG. 59.—Stroke culture of Fraenkel's pneumococcus on blood agar. The colonies are large and unusually distinct. Twenty-four hours' growth at 37° C. Natural size.

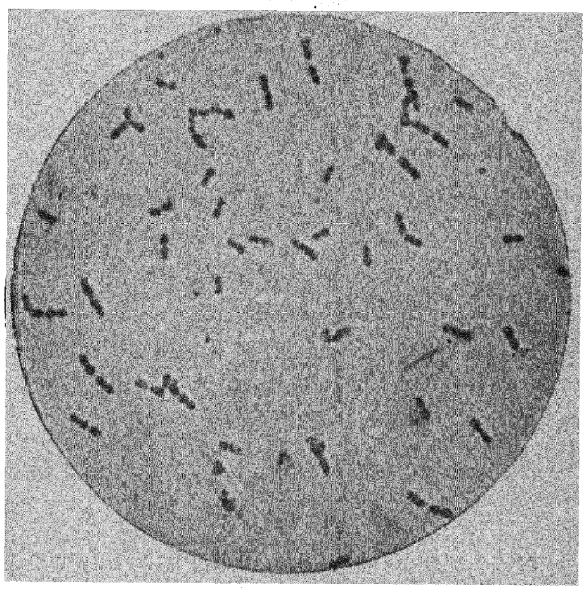


FIG. 60.—Fraenkel's pneumococcus from a pure culture on blood agar of twenty-four hours' growth, some in pairs, some in short chains.
Stained with weak carbol-fuchsin.
×1000.

being suitable) growth forms a slight turbidity, which settles to the bottom of the vessel as a dust-like deposit. On *potato*, as a rule, no growth appears. Cultures may be maintained for long periods, if fresh subcultures are made every two or three days, but they tend ultimately to die out. They sometimes rapidly lose their virulence, so that four or five days after isolation from an animal's body their pathogenic action disappears; but this is not always the case, especially if serum bouillon be used for maintaining subcultures, or if subcultures are made at short intervals. The pneumococcus is comparatively sensitive to external agencies, and in culture soon dies out when dried. When present in dry sputum or blood, however, it is more

resistant and may retain its vitality for a considerable time. A convenient method to preserve the organism is to dry thoroughly the spleen of a mouse dead of pneumococcal septicæmia. In the dried tissue the organism not only remains alive, but retains its virulence comparatively unchanged. In ordinary artificial media pneumococci usually appear as diplococci without a capsule, but in preparations made from the surface of agar or from bouillon, shorter or longer chains may be observed (Fig. 60). After a few days' growth they lose their regular shape and size, and involution forms appear. Usually the pneumococcus does not grow below 22° C., but strains in which the virulence has disappeared often grow well at 20° C. Its optimum temperature is 37° C., its maximum 42° C. It is preferably an aerobe, but can exist without oxygen. It prefers a slightly alkaline medium to a neutral, and does not grow on an acid medium. In ordinary media, as just stated, the pneumococcus does not usually appear to develop a capsule, but as Hiss showed, the absence of a capsule is often only apparent, and if in making cover-glass preparations from such media some serum be used as the diluent, and the films be stained by his copper-sulphate method (p. 111), a capsule can be demonstrated. Capsulation frequently appears in fluid serum media.

When tested on blood agar the pneumococcus is non-hæmolytic, though it forms methæmoglobin, and the colonies are of a greenish colour like those of Str. viridans (p. 241). M'Leod and Gordon have found that it produces a peroxidase leading to the formation of hydrogen peroxide, whilst there is only slight formation of catalase ; the peroxide formed, not being destroyed by catalase, is a factor in inhibiting the growth of the organism. The pneumococcus ferments saccharose, raffinose, and lactose ; a similar fermentative action on inulin is important, as ordinary streptococci do not ferment this sugar. Apparently some samples of inulin are more readily acted on than others. Usually the test is carried out with Hiss's inulin serum water medium, in which coagulation of the serum results (p. 64), but some investigators have had more success with inulin bouillon, acid production being estimated by titration against soda with a phenol-phthalein indicator.

Virulent pneumococci are soluble in bile. To demonstrate this, fresh ox bile autoclaved for twenty minutes at 120° C. and filtered, is added to a well-grown fluid culture (which must be one in simple bouillon) in the proportion of about a fifth of the culture. Two per cent. sodium taurocholate may be similarly used.

Mair recommends as an improvement in the test the use of a 10 per cent., solution of sodium desoxycholate, made distinctly alkaline to phenol-phthalein. Of the solution 0·01 c.c. is added to 5 c.c. of a serum broth culture of the organism; in the case of pneumococcus, clearing of the mixture occurs in ten to fifteen minutes. If the culture is acid, precipitation of the bile acids may occur, but this clears up on the addition of a drop or two of normal caustic soda.

The facts that in cultures the pneumococcus often grows in chains, and that occasionally streptococci are found to develop capsules, have raised the question of the relationship of the pneumococcus to other streptococci. In determining the true pneumococci, biological as well as morphological characters must be studied, and here the bile solubility of the pneumococcus, its failure to produce hæmolysis, and its capacity of fermenting inulin are the important characters. It must be stated, however, as bearing on the close relationships of the pneumococci and streptococci, that Rosenau believes he has succeeded in transforming streptococci into capsulated organisms having all these biological features of the pneumococcus. Morgenroth and his co-workers, by treating pneumococci in various ways by optoquine, have succeeded in effecting transformation into streptococcus viridans and less frequently into a hæmolytic streptococcus; a return to pneumococci was also met with, but only rarely. These results are of importance, and further investigation of the subject is desirable.

Considerable attention has been directed to a group of cocci originally described by Schottmüller, isolated from various disease conditions in man (pneumonia, meningitis, suppurations), which besides possessing voluminous capsules have these surrounded by a viscous material which gives a slimy consistence to cultures and also to pathological exudates. These are related to the pneumococci on the one hand and to the streptococci on the other. The work of the Rockefeller investigators (*vide infra*) suggests that these organisms ought to be classified into two groups. (1) The *Pneumococcus mucosus*. This organism tends to be not so pointed as the ordinary pneumococcus, and its colonies are larger; it is non-hæmolytic on blood agar, soluble in bile, gives rise to acid and clot in Hiss's inulin serum water, and is very pathogenic to white mice and rabbits. Antisera produced by strains of this coccus, while showing cross agglutination towards members of their own group, do not agglutinate streptococci and usually also not other pneumococci. (2) The *Streptococcus mucosus*. This organism is generally round, occurs in chains, and the colonies are less transparent than those of the

pneumococcus; it is usually non-hæmolytic, is not soluble in bile, does not ferment inulin, and is less pathogenic to mice than the last. Thus while the pneumococcus mucosus is practically a true pneumococcus, the streptococcus mucosus forms a connecting link with the true streptococci. It has also been found that the mucous capsules may disappear on animal passage and may thus represent a reaction of the organism developed under special conditions in the original host (Browning and Gulbransen).

Experimental Inoculation.—The *pneumococcus* of Fraenkel is pathogenic to various animals, though the effects vary somewhat with the virulence of the race used. The susceptibility of different species, as Gamaleia showed, varies to a considerable extent. The rabbit, and especially the mouse, are very susceptible; the guinea-pig, the rat, the dog, and the sheep occupy an intermediate position; the pigeon is immune. In the more susceptible animals the general type of the disease produced is not pneumonia, but a general *septicæmia*. Thus, if a rabbit or a mouse be injected subcutaneously with pneumonic sputum, or with a scraping from a pneumonic lung, death occurs in from twenty-four to forty-eight hours. There is some fibrinous infiltration at the point of inoculation, the spleen is often enlarged and firm, and the blood contains capsulated pneumococci in large numbers (Fig. 61).

When relatively avirulent cultures are used, local inflammatory changes are set up in susceptible animals instead of septicæmia, and corresponding results are said to follow when virulent cultures are injected into such animals partially immunised. So also in the more resistant animals, such as the sheep and the dog, the lesions produced are of inflammatory type, and when the injections are made into the lungs pneumonia may be set up. Lamar and Meltzer by intra-bronchial insufflation of cultures of pneumococci in the dog, succeeded in producing typical lobar pneumonia; although, in the cases where recovery followed, the general course of the infection did not fully correspond with that in the human subject. Comparatively large amounts of culture were used. The most conclusive experiments on the subject, however, are those of Blake and Cecil who, by intra-tracheal injection in monkeys of virulent pneumococci in minute doses, were able regularly to set up a lobar pneumonia which ran a course like that of the human disease, recovery taking place by crisis. On the other hand, the introduction in larger amounts of similar pneumococci into the throat or nose of these animals was not followed by pneumonia, even though the

organisms persisted for a considerable time. This is of importance in connection with the natural occurrence of pneumonia, as it points to some additional factor being necessary for its origin, especially in view of the fact that monkeys are rather more susceptible than the human subject. Blake and Cecil met with spontaneous pneumonia in monkeys kept in confinement, and the disease, which had the same features as that experi-

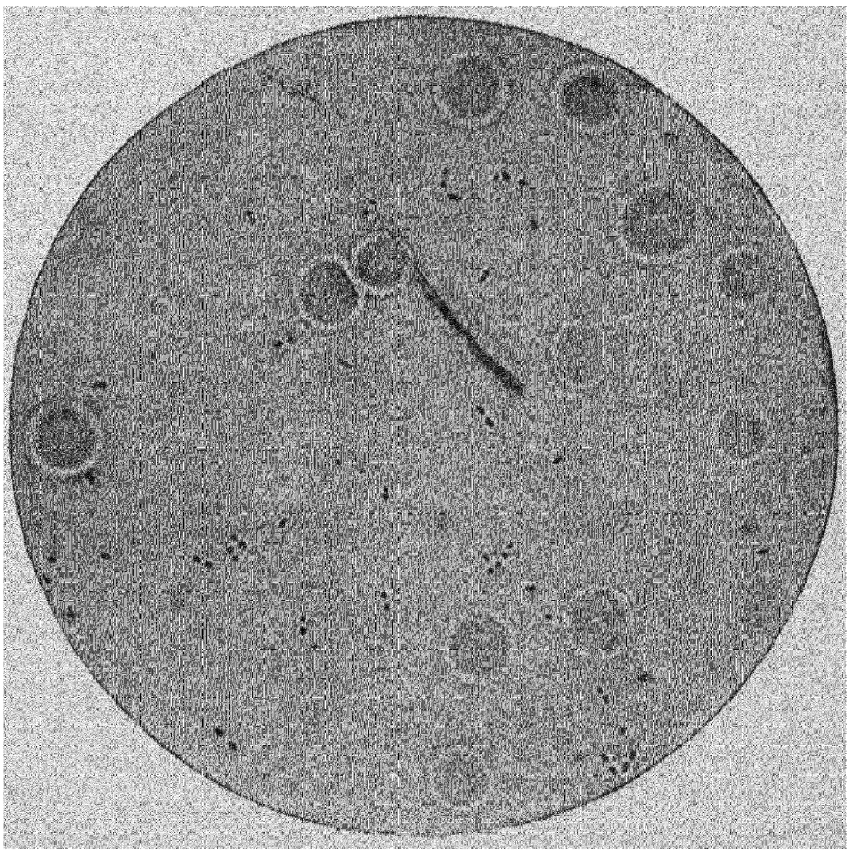


FIG. 61.—Capsulated pneumococcus in blood taken from the heart of a rabbit, dead after inoculation with pneumonic sputum.
Dried film, fixed with corrosive sublimate. Stained with carbol-fuchsin and partly decolorised. ×1000.

mentally produced, readily passed from animal to animal. They came to the conclusion that when virulent pneumococci are introduced into the trachea they probably penetrate the wall of a large bronchus near the root of the lung, and thence spread by the interstitial tissue outwards from the hilum; a general inflammation of the substance of the lung is thus produced. They were unable to set up lobar pneumonia in monkeys by subcutaneous or by intravenous injection; on the other hand, secondary invasion of the blood stream by the pneumococci

occurred in the experimentally produced lobar pneumonia, the number of organisms increasing in cases which terminated fatally. Gaskell, using adjusted doses by the intratracheal method, has recently found that the types of pneumonia thus produced in the rabbit varied according to the virulence; with a certain strain a lobular pneumonia may result, while with one of higher virulence there is lobar consolidation.

A fact which at first appeared rather to militate against the pneumococcus being the cause of pneumonia was the discovery by Pasteur and others of this organism in the saliva of healthy men. It can certainly be isolated by inoculation of susceptible animals, from the mouths of a considerable proportion of normal men, from their nasal cavities, etc., being probably in any particular individual more numerous at some times than at others (especially, it is stated, during the winter months). It must be noted, however, that according to the Rockefeller investigations (*infra*) the pneumococcus occurring in the healthy naso-pharynx is usually of Type IV., *i.e.* belongs to the group least pathogenic to man. The more pathogenic types are found almost exclusively in the mouths of convalescents and of contacts and in rooms where pneumonia cases have been nursed. While these types usually rapidly disappear from convalescents and contacts they may persist long enough to justify the view that certain persons may act as carriers of the disease. The exact conditions leading to actual infection are imperfectly understood, but we must recognise the importance of predisposing causes in the etiology of the disease, as in the case of the diseases caused by pyogenic staphylococci, streptococci, the bacillus coli, etc. By such causes the vitality and power of resistance of the lung may be diminished, and then the pneumococcus gains an entrance. We can therefore understand how such factors as cold, alcoholic excess, abnormal conditions of the respiratory tract,—a slight bronchitis,—etc., can play an important part in the causation of pneumonia.

The Occurrence of the Pneumococcus in Pneumonia and other Conditions.—The pneumococcus occurs in every variety of the disease—in acute croupous pneumonia, in bronchopneumonia, in septic pneumonia. In a case of croupous pneumonia the pneumococci are found all through the affected area in the lung, especially in the exudation in the air cells. They also occur in the pleural exudate, and in the lymphatics of the lung. The greatest number are found in the parts where the inflammatory process is most recent, and therefore such parts are preferably to be selected for microscopic examination, and

as the source of cultures. When the inflammation is resolving, some of the organisms often stain badly (*e.g.* tend to lose the Gram-positive reaction); such individuals are probably either dead or dying. Sometimes there occur in pneumonic consolidation areas of suppurative softening, which may spread diffusely. In such areas the pneumococci occur with or without ordinary pyogenic organisms, streptococci being the commonest concomitants. In other cases, especially when the condition is secondary to influenza, gangrene may supervene and lead to destruction of large portions of the lung. In these a great variety of bacteria, both aerobes and anaerobes, are to be found. By direct extension to neighbouring parts, empyema, pericarditis, and lymphatic enlargements in the mediastinum and neck may take place; the pneumococcus may occur either alone or with pyogenic cocci.

In a considerable proportion of cases of lobar pneumonia, pneumococci can be cultivated from the blood. This represents a bacteriæmia, due to a secondary extension of organisms from the lung, and varies in degree in different cases. When the number is considerable the effects are liable to be severe; according to some observers a number of over fifteen cocci per c.c. is of fatal import. It is interesting to note that in the lobar pneumonia experimentally produced by Blake and Cecil in monkeys, a progressive increase of pneumococci in the blood was accompanied by a progressive diminution in the number of leucocytes and led to a fatal result. The presence of pneumococci in the blood explains the occurrence of inflammatory complications in pneumonia such as meningitis, suppurations in connective tissue, joints, etc. (A primary meningitis, apart from pneumonia, may also be produced by pneumococci (p. 296).) Ulcerative endocarditis may also develop, sometimes after the occurrence of a distinct crisis, and in this connection we may refer to the interesting observation made by Mair, namely, that in rabbits partially immunised by dead cultures intravenous injection of virulent pneumococci is followed by endocarditis in a large proportion of cases. The pneumococci obtained from the vegetations were found to have lost their virulence.

Other Types of Pneumonia.—The so-called "simple" or non-suppurative type of broncho-pneumonia common in children shows certain points of difference from the lobar type. It represents a pneumonia starting as multiple foci, usually in both lungs, and is the result of spread of inflammatory change from the terminal bronchioles along the air passages to the

alveoli, although extension through the walls of the bronchioles also takes place. There is an absence of the rapid general involvement of the interstitial tissue of the lung seen in lobar pneumonia, and an important point is that it is characterised by the absence of crisis. In broncho-pneumonia in children the pneumococcus is again the important causal agent and can be found in the vast majority of cases. Other organisms of the pyogenic group may occur along with it or sometimes alone. In the adult the proportion of cases caused by organisms other than pneumococcus is somewhat greater. In the broncho-pneumonia secondary to diphtheria the pneumococcus may be accompanied by the diphtheria bacillus and also by pyogenic cocci. In typhoid pneumonias the typhoid bacillus or the B. coli may be alone present or may be accompanied by the pneumococcus. In septic pneumonias the pyogenic cocci in many cases are the only organisms discoverable, but the pneumococcus also may be present. In influenza there is a general lowering of the resistance of the bronchial mucosa and a great variety of organisms, including influenza bacillus, pneumococcus, streptococcus, staphylococcus, and M. catarrhalis, etc., are enabled to extend to the lower bronchial passages. The bacterial flora present in the broncho-pneumonic lesions therefore varies considerably, but pneumococci and influenza bacilli are predominant and are often present together. In very severe influenza, such as occurred in 1918, in addition to broncho-pneumonia, lobar pneumonia due to the pneumococcus occurred, and sometimes even a true septicæmia due to this organism—an indication of remarkably lowered resistance. We may note that empyema due to the pneumococcus is more frequent in children than in adults, being a not uncommon sequel of broncho-pneumonia.

It is difficult to explain why sometimes the pneumococcus is associated with a spreading inflammation as in croupous pneumonia, whilst at other times it is chiefly localised in the catarrhal patches in broncho-pneumonia. It is quite likely that in the former condition the organism is possessed of a different order of virulence, and probably the susceptibility of the lung tissue varies at different periods of life—the resulting lesion, of course, depends on both of these factors. We have, however, a closely analogous fact in the case of erysipelas, this being a spreading lesion produced by a streptococcus which, when less virulent, causes only local inflammatory and suppurative conditions. As already mentioned, the existence of a crisis in lobar pneumonia, indicating a rapidly developed

immunity, is a characteristic feature, and the association of this with a rapid growth of the organisms throughout the framework of the lung has undoubtedly an important significance. When the organisms are mainly present within the air vesicles and bronchioles, as in broncho-pneumonia, this rapid immunity reaction is not met with.

In children the pneumococcus may extend along the Eustachian tubes and give rise to otitis media, this being a fairly frequent and important lesion; thence it may spread to the brain and give rise to leptomeningitis. It may be noted that next to the lungs the meninges are the parts most liable to attack by the pneumococcus, usually secondary to a form of pneumonia or otitis media, but sometimes as a primary occurrence (p. 296). Other primary pneumococcal lesions (apart from pneumonia) such as arthritis, peritonitis, etc., are also met with. Not infrequently rhinitis is due to pneumococci, as are also inflammatory and suppurative lesions in the antrum and frontal sinuses. Conjunctivitis is sometimes caused by pneumococci (p. 261), as is also corneal ulcer.

Summary.—We may accordingly summarise the facts regarding the relation of Fraenkel's pneumococcus to the disease by saying that it can be isolated from nearly all cases of acute croupous pneumonia, and also from a considerable proportion of other forms of pneumonia. When introduced into the trachea in suitable animals it gives rise to lobar pneumonia. We are therefore justified in holding that it is the chief factor in causing croupous pneumonia, and also plays an important part in other forms.

Immunity Phenomena.—Lobar pneumonia represents an inflammatory lesion accompanied by the symptoms of an acute poisoning. In very few cases does death take place from the functions of the lungs being interfered with to such an extent as to cause asphyxiation; it is through cardiac failure, from great interference with the heat-regulating mechanism, and from general nervous depression that death usually results. These considerations, taken in connection with the fact that the organisms are found in the greatest numbers in the lung, suggest that a toxic action is at work. We may say, however, that attempts to separate specific soluble toxins have failed, and that the poisonous substances are apparently of the type of endotoxins set free by lysis and of similar nature to those formed by various other bacteria. In conformity with this, it is found that whilst active immunity can be readily obtained, there is no evidence that it depends upon specific antitoxins;

it is *antibacterial*, and is due to the development of powers to destroy the invading organisms.

Animals can be immunised against the pneumococcus by inoculation with virulent cultures killed by heating at 55° C., with autolysed cultures or with cultures which have become attenuated in various ways. Sometimes one or two injections, at intervals of several days, are sufficient for immunisation, but the immunity has been observed to be usually of a fleeting character and may not last more than a few weeks ; a process of intensive and rapid immunisation is described below. The serum of such immunised animals neutralises the action of pneumococci in susceptible animals when added to the organisms ; it protects also against subsequent inoculation with a certain dose of pneumococci, and if injected within twenty-four hours after inoculation, may even prevent death.

Pneumococcus infection is, however, one of the conditions where a more effective immunity is developed by inoculating with living than with dead organisms. Neufeld and Rimpau insisted that the ultimate use of living virulent cultures was necessary for the attainment of a high degree of immunity. Blake and Cecil in their work on experimental pneumonia in monkeys likewise found that small doses of living virulent pneumococci or larger doses of the same organisms in an attenuated state protected against intratracheal injection of virulent pneumococci, whilst vaccines consisting of dead organisms failed to do so. The latter, however, modified the secondary invasion of the blood by pneumococci when pneumonia developed, and also afforded protection against experimental pneumococcal septicæmia. There was thus a humoral immunity without protection against intratracheal infection. They point out, however, that these results do not mean that dead vaccines may not protect the human subject against pneumonia under natural conditions, seeing that the monkey is more susceptible. As a matter of fact, we have evidence that dead vaccines do afford a degree of protection. The use of the living virulent organism is, of course, difficult to control, and is impracticable as a preventive measure against natural pneumonia in the human subject.

The protective potency of an anti-pneumococcic serum as measured by the number of fatal doses of virulent pneumococci against which a given amount of serum will protect an animal (*e.g.* a mouse), may reach a high degree. In studying the development of antibodies in the blood, Neufeld and Händel found that there may be little antagonistic effect till a certain

concentration of these is reached, whilst beyond this point the antibodies may produce a rapid destruction of a large number of pneumococci. It is to be noted further that the simple arithmetical proportion seen in relation to toxin and antitoxin does not obtain in the case of these bacterial antibodies; if a given amount of antiserum protects against a given amount of organisms, it does not follow that half that amount of serum will protect against half the amount of organisms. Armstrong has recently studied quantitatively the development of pneumococcic antibodies and has found, for example, after a single dose of dead vaccine, that there is an inductive phase of about three days, after which for two or three days there is an access of antibodies to the blood at a rapidly increasing rate, corresponding to a geometrical progression. The increase then becomes less rapid and the maximum concentration occurs about the eighth day. He finds further that in the case of an attack of lobar pneumonia, running an ordinary favourable course, similar phenomena are met with. A rapid outpouring of antibodies occurs about the fifth day, and the content in the serum increases till about the time of the crisis, after which it remains high for some time. We thus have the picture of a rapid development of active immunity, which on reaching a certain point results in a rapid destruction of the organisms.

An anti-pneumococcic serum contains the various types of antibodies already mentioned (p. 195), including precipitins, the actions of which are demonstrable against an autolysed culture. The pneumococcus, however, belongs to the group of organisms which are not killed directly by normal serum, and further, there is no evidence that an antiserum, in virtue of its immune-bodies leading to the fixation of complement, possesses bactericidal action. The organism is sensitive, however, to opsonic action, and thus an antiserum may exert its beneficial action by means of its opsonins (bacteriotropins, as they were called by Neufeld). Observations have been carried out on the opsonic properties of the serum in cases of pneumonia, and it has been found that towards the time of crisis in favourable cases the opsonic index rises distinctly, and after defervescence gradually returns to normal. Such increase, however, does not appear to be comparable with the marked rise in the protective property of the serum, and, at present, the essential phenomena cannot be taken as entirely due to opsonic action. Blake and Cecil in their work on experimental pneumonia in monkeys did not consider it possible to explain the immunity by the antibodies demonstrable in the serum, and considered that some

other factor, such as changes locally in the tissues, might be concerned in the immunity reaction and the destruction of the organisms.

The leucocytosis in pneumonia has long been recognised, and when present in distinct degree indicates that a satisfactory cellular response is taking place. It, however, scarcely indicates more than this, and certainly does not promise recovery, as death may occur in a variety of ways. On the other hand, the absence of leucocytosis, or its disappearance after being present, is undoubtedly of unfavourable omen. Whilst leucocytosis is of reactive nature it does not explain immunity; it is simply due to the presence of chemotactic substances in the blood, such as are present in numerous pathogenic infections and, as we have indicated, its significance is that the bone-marrow is reacting satisfactorily.

A substance derived from the infecting pneumococcus sometimes appears in the urine during pneumonia. It gives a precipitin reaction with the antiserum corresponding to the type of pneumococcus causing the infection, and can be detected by mixing equal quantities of clear centrifuged urine with an equal amount of antiserum; this method can, in fact, be used for determining the type of pneumococcus present in the body. The appearance of this substance in the urine is an indication that the case is a severe one, and a progressive increase in amount is a bad prognostic sign.

It may be noted here, in conclusion, that in man immunity against pneumonia may be short-lived, as in many cases of pneumonia a history of a previous attack is elicited.

The difficulty of interpreting the various serological facts observed in pneumonic conditions led Lamar to investigate the action of certain chemical bodies, belonging to the soaps, on pneumococci. Welch long ago observed changes in the protoplasm of pneumococci in pneumonic exudates, pointing to the occurrence of lysis. Lamar has found that pneumococci treated with sodium oleate and especially with potassium soaps of acids having a high iodine value—*e.g.* linoleic and linolenic acids—undergo morphological changes and become more subject to autolysis and more sensitive to the lytic action of sera, the latter being especially evident when immune sera are employed. The action of the soap is probably exerted on the lipoidal moiety of the bacterial cells, which are thus rendered more pervious to the serum constituents. There is evidence, however, that the protein constituents of sera exercise an inhibitory effect on the lytic action of the soaps, and Lamar made the interesting observation that this inhibitory action can to a certain extent be neutralised by the use of boric acid. These observations have a bearing on the explanation of natural recovery from such

infections since in inflammatory exudations soaps form a definite constituent.

Differentiation of Strains of the Pneumococcus by Antisera.—The possibility of effecting this is one of the most important consequences of the study of immunity against the pneumococcus. It had been long recognised that strains of the pneumococcus derived from different sources present individual peculiarities, but it was not till the recent exhaustive investigation of the subject in the Rockefeller Institute, New York, that definite results were obtained. In the study of the agglutinating and protecting properties of antisera prepared by inoculating animals against a long series of cultures isolated from cases of acute lobar pneumonia, it was proved that sera derived from certain strains, on the one hand, would almost indiscriminately agglutinate some of these strains, and, on the other, had little or no effect on other strains. It was further found that the agglutinating and protective qualities of these sera were parallel. In this way it was possible to group the strains under four types. Three of them (I., II., III.) were definite, and a fourth (IV.) was formed of strains in which an antiserum usually only agglutinated the strain which originated it, and had little or no capacity of agglutinating the strains of Types I., II., III. The members of Type III. could be recognised not only by their originating agglutinating sera specific to the group, but presented cultural features which characterised them as the *Pneumococcus mucosus* (see p. 273). Types I. and II. between them accounted for 60 per cent. of the cases of pneumonia studied and are of relatively high virulence for man, this being specially the case with Type II. Type III., while accounting for only 12 per cent. of cases, is of highest virulence, the mortality with it being 45 per cent. Type IV. was found in 24 per cent. of cases and caused the lowest mortality (16 per cent.); the strains occurring in the mouth of healthy individuals probably belong to this type. It is probable, however, that in different parts of the world different strains prevail. Thus, in South Africa, Lister found that, while the New York Types I. and II. were common, nearly a third of all cases of pneumonia were associated with another type which apparently did not occur to any extent in New York. Davidson and McLachlan have found that among a variety of pneumococcal infections (studied in Edinburgh) Type I. predominates and is present in 64 per cent. of all cases, while Type II. is relatively infrequent (7 per cent.). Types III. and IV. represented respectively 15 per cent. and 14 per cent. of the infections.

Methods of classifying Pneumococci by Agglutination.—This depends on the observer being furnished with the type sera (I., II., III.) of the Rockefeller Institute. A white mouse is inoculated intraperitoneally with 0·5 to 1 c.c. of a saline emulsion of a bean-sized piece of sputum, freed from surface contamination by washing in sterile saline. The mouse may die in from five to twenty-four hours, and if the peritoneal exudate contains a strong and fairly pure growth of the pneumococcus the abdominal cavity is washed out with 5 c c. saline, cultures being at the same time made in broth and on blood-agar plates. The peritoneal washings are first centrifuged slowly to precipitate gross material, and the supernatant fluid is then centrifuged at a high speed to precipitate the bacteria. The bacterial deposit is suspended in saline to form a fairly heavy suspension which is used for a macroscopic sedimentation test. If the pneumococci in blood cultures or in exudates are to be employed, suspensions may be obtained by similar procedures. A bacterial emulsion being prepared, 0·5 c.c. of Serum I. (1 : 20), 0·5 c.c. of Serum II. (undiluted), 0·5 c.c. of Serum II. (1 : 20), and 0·5 c.c. of Serum III.[1] (1 : 5) are placed in four tubes, and 0·5 c.c. bacterial emulsion added to each, and in a fifth tube a mixture of 0·1 c.c. sterile ox bile and 0·4 c.c. bacterial emulsion is made up ; the series is placed in a water bath at 37° C. for one hour, and the result read. Sedimentation in any one of the four tubes indicates that the strain belongs to the type by the serum of which it is agglutinated ; if no reaction occurs in any of the tubes, and the organism is soluble in bile, it belongs to Type IV.

The Treatment of Pneumonia with Antisera.—Many years ago the Klemperers treated a certain number of cases of human pneumonia by serum derived from immune animals, apparently with a certain measure of success, and afterwards Römer issued through Merck a polyvalent serum prepared by immunising different species of animals with growths of the pneumococcus on sheep-serum glycerine-bouillon and mixing their sera. The results obtained, though in some cases satisfactory, were irregular, and Neufeld and Händel then insisted that it was essential that the antiserum used should correspond to the particular strain present in the case to be treated.

Evidence confirming this view has been obtained in the New York investigations on pneumonia, in which the determination of the different types of the pneumococcus was followed by an estimate of the therapeutic capacities of the antisera prepared against Types I., II., III. (*vide supra*). It was found that while the antiserum to Type I. had a curative effect on cases of pneumonia due to the Type I. pneumococcus, the antisera to Types II. and III. had practically no effect on cases attributable

[1] There is apparently sometimes difficulty in effecting the agglutination of Type III. on account of the consistence of its capsule, and special methods may be necessary ; see Hanes, *Journ. Exp. Med.*, 1914, xix. 38.

to these types; furthermore, the antiserum to Type I. had little or no effect on cases caused by Types II. and III. These facts throw light on the irregular and generally disappointing result obtained hitherto with the ordinary polyvalent anti-pneumococcal sera.

The Rockefeller serum is prepared by immunising horses first with dead cultures; daily injections are given for six days, followed by an interval of a week, then six further daily injections are given; it is sometimes necessary to follow these up by the use of living organisms. Uniformity of strength in successive sera thus prepared is secured by determining the largest amount of an eighteen hours' culture against which 0·2 c.c. of the serum will protect a white mouse —a comparison with the effects of the same amount of a standard serum being at the same time made.

In the therapeutic application of the serum in a case of Type I. pneumonia large quantities must be used, and it is therefore a necessary preliminary to determine whether the patient exhibits hypersensitiveness to horse serum, and to desensitise him if this exists (see chapter on Immunity). If the way be clear, the serum, diluted with an equal amount of sterile saline made with freshly distilled water, is administered by the intravenous method—10–15 c.c. being given at the rate of 1 c.c. per minute—changes in the heart's action and in respiration and the occurrence of urticaria being watched for, and the treatment being suspended for a quarter of an hour if untoward symptoms seem to increase; if this does not occur, the remainder of the first dose may be given during fifteen minutes. The initial dose should be from 90–100 c.c., and the injections ought to be repeated every eight hours till about 250 c.c. serum have been given. Very soon after commencement of treatment the temperature may rise, but in many cases this is quickly succeeded by a fall, with improvement in the patient's general condition, stoppage of extension of the lung lesion, and prevention of invasion of the blood by the pneumococci. The effects of the treatment so far have been satisfactory—of 107 cases treated in the Rockefeller Institute Hospital up to October 1917 only 7·5 per cent. died, as compared with a mortality of 25 to 30 per cent. in cases of Type I. pneumonia before the serum treatment was introduced. Up to the present no means of treating pneumonia of Types II. and III. by serum methods have been found practicable, and, as has been stated, the pneumococci of Type IV. do not yield a group antiserum.

Prophylactic Vaccination.—In the South African mines a special situation existed in consequence of the great susceptibility to pneu

monia occurring in the native labourers, who are chiefly recruited from subtropical regions. As the case incidence ran from 30 to 150 per thousand per annum, and the mortality from 10 to 30 per thousand, the disease is a very serious one. Almroth Wright introduced prophylactic vaccination, and Lister, founding on his investigations (*vide supra*), prepared a vaccine containing the three prevalent types of the pneumococcus. In the latest applications of the method three injections at seven-day intervals of, in all, 7000 million cocci, killed by an antiseptic, were administered. A very marked diminution in the incidence of the disease followed.

Methods of Examination.—In stained films of sputum, pus, or other exudate containing pneumococci, the outstanding feature is the predominance of diplococcal forms the elements of which may have a lanceolate shape and which are Gram-positive. Often a capsule stain demonstrates the capsule in such material, and it may even appear stained in Gram films. Cultures on blood agar should be made which after twenty-four hours at 37° C. will, if the pneumococcus be present, show characteristic colonies. Subcultures on serum bouillon or serum-smeared agar will show capsulation. Bile-solubility and reaction with inulin may be tested; a white mouse may be inoculated to test the pathogenicity and to afford in blood films corroborative evidence of capsulation.



FIG. 62.—Friedländer's pneumobacillus, showing the variations in length, also capsules. Film preparation from exudate in a case of pneumonia. ×1000.

The Pneumobacillus (*Encapsulatus pneumoniæ*).—This organism is of historic interest, as it was the first organism described in pneumonia, though there is little doubt that in early days it was often confused with the pneumococcus. It does not occur alone in more than about 1 per cent. of cases of pneumonia; in the pneumonia caused by it the consolidated lung has often a somewhat slimy or mucoid character. In the sputum it may appear as a very short diplobacillus possessing a capsule, but it also frequently is seen in the form of long rods (Fig. 62). It stains by ordinary methods, but is Gram-negative.

It can be easily isolated on agar plates, on which it forms large whitish moist viscid colonies (cf. B. lactis aerogenes of B. coli group).

In gelatin stabs, at the site of the puncture the growth is sometimes heaped up above the level of the medium, and along the needle tract there is a white granular appearance (Fig. 63). The gelatin is not liquefied. The organism grows well on all ordinary media, and on these its bacillary nature is in marked evidence (Fig. 64). It may form capsules in serum bouillon. It is non-motile. There is some variation among different strains as regards their fermentative reactions. Certain strains conform in their biochemical characters to the inosite fermenting sub-group of the coliform bacilli (*vide* p. 405 and table p. 442) and ferment glucose, lactose, dulcite, saccharose, adonite, and inosite, with gas production. Some strains, however, fail to ferment lactose. It usually can form indol from peptone. The pneumobacillus is closely related to the B. coli.

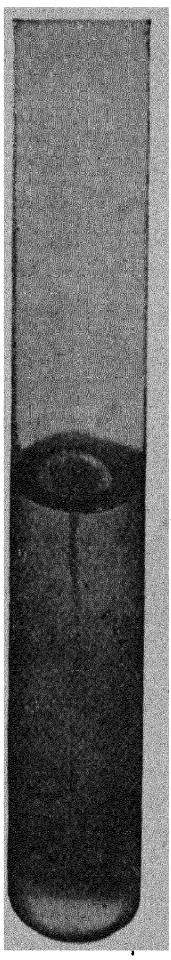


FIG. 63.—Stab culture of Friedländer's pneumobacillus in gelatin, showing the nail-like appearance; ten days' growth. Natural size.

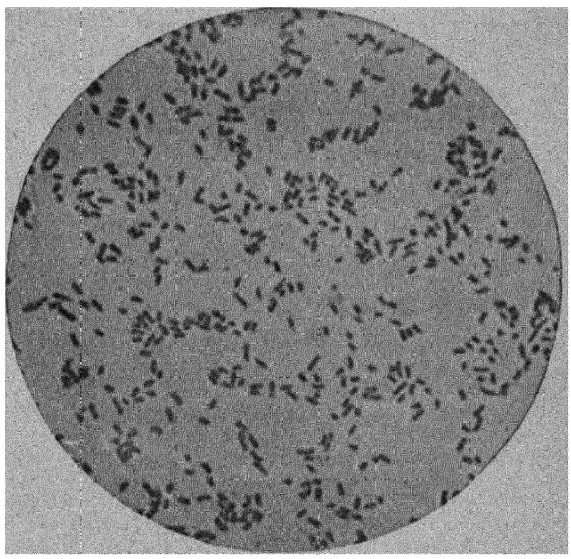


FIG. 64.—Friedländer's pneumobacillus,[1] from a young culture on agar, showing some rod-shaped forms. Stained with thionin-blue. ×1000.

When injected into mice and guinea-pigs it causes a septicæmia and can be seen in the heart blood to possess a capsule. It is less pathogenic to rabbits and dogs, but when injected into the trachea in these animals it originates a pneumonia. As stated above, it is the only organism present in a small number of cases of human pneumonia, and it has also been isolated from

[1] The apparent size of this organism, on account of the nature of its sheath, varies much according to the stain used. If stained with a strong stain, *e.g.* carbol-fuchsin, its thickness appears nearly twice as great as is shown in the figure.

conditions of empyema, meningitis, appendicitis, and pyæmia; a bacillus closely related has been found in rhinoscleroma (*q.v.*). It is a not infrequent inhabitant of the mouth and nose of healthy individuals. From its historical associations an altogether undue importance has been attached to this bacillus.

Epidemic Cerebro-Spinal Meningitis or Cerebro-Spinal Fever

As the result of observations on this disease in different parts of the world, it has been now established that the causal agent is the *Diplococcus intracellularis meningitidis*, first described by Weichselbaum, and now usually known as the *Meningococcus* (*Neisseria intracellularis*). This organism is a small coccus measuring about 1 μ in diameter; it usually occurs in pairs, the adjacent sides being somewhat flattened against each other. In most cases the cocci are chiefly contained within polymorphonuclear leucocytes in the exudation (Fig. 65); in some cases, however, the majority may be lying free. It stains readily with basic aniline dyes, but is Gram-negative. Both in appearance and in its staining reactions it is closely similar to the gonococcus (*vide* p. 299). The organism can readily be cultivated outside the body, but the conditions of growth are somewhat restricted —"trypagar" (p. 55), agar with an admixture of serum, ascitic fluid, or blood (p. 54) or boiled blood digest agar, is to be recommended. The optimum reaction is one neutral to phenolphthalein. Growth takes place best at the temperature of the body, and practically ceases at 25° C. On these media the colonies are circular discs with a slightly opaque centre fading into a delicate transparent margin (Fig. 66), and they have a smooth, shining surface; they have a slightly mucoid consistence and readily emulsify in water or normal saline. When

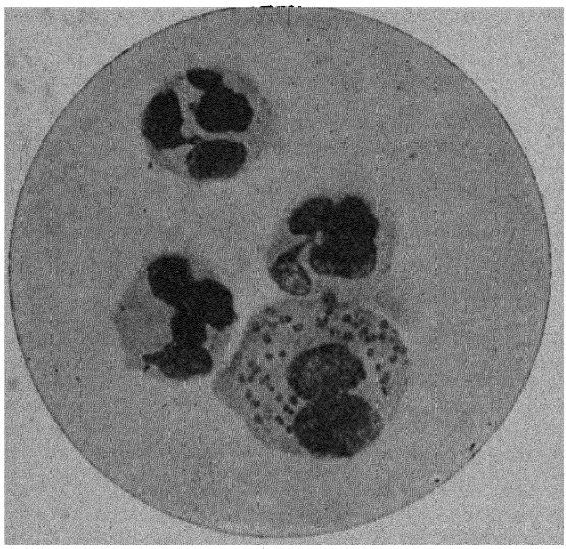
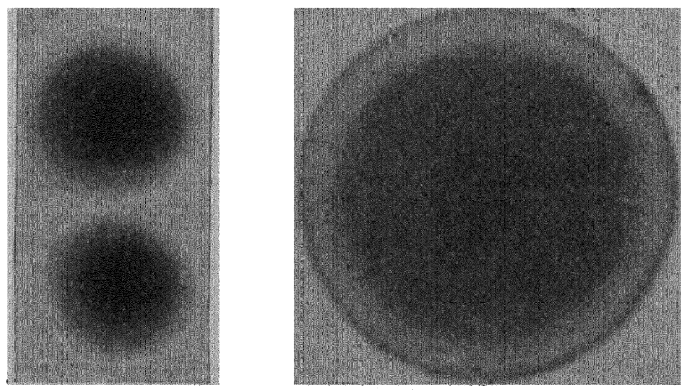


Fig. 65.—Film preparation of exudation from a case of meningitis, showing the meningococci within leucocytes. See also Plate I., Fig. 3.
Stained with carbol-thionin-blue. ×1000.

examined under a low magnification the centre appears somewhat yellowish, and the margins usually are smooth and quite regular ; at a later period of growth slight crenation may appear, especially when the medium is somewhat dry. The colonies may be of considerable size, reaching sometimes a diameter of 2 to 3 mm. on the second day. Variations in appearance are met with, and Atkin has related these to different serological types. A stroke culture gives a broad line of growth of similar character ; the margins tend to be somewhat crenated, and isolated colonies often occur. On plain agar the colonies are very much smaller, and sometimes no growth occurs ; subcultures especially often fail to give any growth on this medium.



a *b*

FIG. 66.—*a*. Two-day colonies of the meningococcus on Martin's medium (p. 55), ×9 ; *b*. the same, in which illumination has been arranged to show finely granular centre and transparent margin, ×12. Compare with Fig. 69.

From photographs by the late Prof. W. B. M. Martin.

In serum bouillon the organism produces a general turbidity with formation of some deposit after a day or two.

In the preparation of a medium suitable for culture purposes most attention has been directed to the readiness with which growth takes place and to the abundance of the growth, but, as pointed out by Murray and Ayrton, retention of the virulence of the organism should also be a criterion of suitability. They have found that retention of virulence depends upon substances contributed by the digest used as a basis, and they have also made the interesting observation that the addition of an extract of polymorpho-nuclear leucocytes raises the virulence *in vitro*. (For the formulæ of their media their original papers must be consulted.)

The meningococcus ferments maltose and dextrose with acid production, a property which distinguishes it from the micrococcus catarrhalis (*vide infra*); it has no action on saccharose. Fermentation tests can be carried out by means of either fluid or solid media containing 1 per cent. of the sugar to be tested, along with neutral-red or litmus as an indicator (p. 63). In cultures the organism presents the same appearance as in the body, and often shows tetrad formation. There is also a great tendency to the production of involution forms (Fig. 67), many of the cocci becoming much swollen, staining badly, and afterwards undergoing disintegration. This change, according to Flexner's observations, would appear to be due to the production of an autolytic enzyme, and he has also found that this substance has the property of producing dissolution of the bodies of other bacteria. The life of the organism in cultures is a comparatively short one; after a few days cultures will often be found to be dead, but, by making subcultures every three or four days, strains can be maintained alive for considerable periods. On egg medium (p. 56), however, it survives for a considerable time. The organism is readily killed by heat at 60° C., and it is also very sensitive to weak antiseptics; drying for a period of a day has been found to be fatal to it. The facts established accordingly show it to be a somewhat delicate parasite.

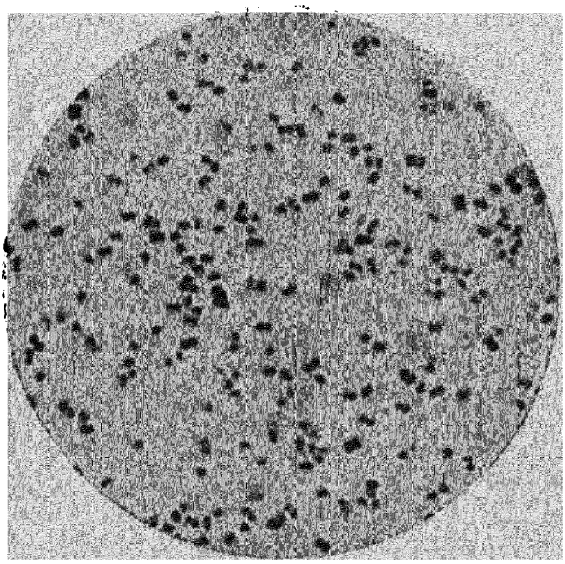


FIG. 67.—Pure culture of Diplococcus intracellularis, showing involution forms.

As stated above, the organism occurs in the exudate in the meninges and in the cerebro-spinal fluid, and it can usually be obtained by lumbar puncture. In acute cases, especially in the earlier stages, it is usually abundant; but in the later stages of cases of more subacute character, its detection may be a matter of difficulty, and only a few examples may be found after a prolonged search. But it should be recognised that *at any stage* microscopic examination and even cultivation may sometimes give a negative result. In most cases the lesions are

practically restricted to the nervous system, but occasionally complications occur, and in these the organism may be present. It has been found, for example, in arthritis, pericarditis, pneumonic patches in the lung, and in other inflammatory conditions associated with the disease, and also occasionally in purpuric patches in the skin and stomach, though the ordinary petechial eruption seems to be of toxic origin. In a small proportion of cases it may be obtained from the blood during life.

Experimental inoculation shows that the ordinary laboratory animals are relatively insusceptible to this organism. An inflammatory condition may be produced in mice and small guinea-pigs by intraperitoneal injection, and a fatal result with symptoms of collapse may follow. The results vary according to the virulence, but often the organism does not undergo very active multiplication, though it may sometimes be cultivated from the blood; none of the lesions in the nervous system are reproduced Similar results are produced by the endotoxin in dead cultures, and occasionally the lethal dose of the dead organisms may equal that of the living (Gordon). There is thus evidence that an active endotoxin plays an important part in the pathology of the disease Flexner and also Stuart M'Donald have shown that cerebro-spinal meningitis may be produced in monkeys by injections of the organism into the spinal canal, the latter observer finding that exudate containing meningococci was more effective than cultures In such experiments the organism extends upwards to the brain, and produces meningitis within a very short time. The resulting lesions, both as regards their distribution and general characters, and also as regards the histological changes, resemble the disease in the human subject. Even these animals, however, are manifestly less susceptible than the human subject.

The meningococcus can usually be found in the naso-pharynx of patients suffering from the disease, and there is no doubt that this is the usual channel of infection. In cases where recovery occurs, the organism may persist there for a varying period of time—usually only for a week or two, but sometimes for months. There is difference of opinion as to the route by which the organism passes from the naso-pharynx to the meninges. One view is that it passes directly by the lymphatics to the base of the brain, but satisfactory evidence of this is wanting. The other view is that it passes by the blood stream; this is in accordance with what occurs in other infections, and is also supported by the fact that in some very acute cases with purpuric eruption, it has been found in the blood before meningeal symptoms have appeared, and also occasionally in septicæmic types without meningitis. For a considerable time it has been known that contacts with cases of cerebro-spinal fever

often harbour the meningococcus in the naso-pharynx, that is, are "carriers," and during the war this subject was extensively investigated. In fact, the examination of contacts became a routine procedure. The percentage of "positives" amongst contacts varies; sometimes it has been found to be twenty or even higher. Non-contacts also have been examined during epidemics, and amongst them also a considerable proportion, though not so great as amongst contacts, have been found to be carriers. In some carriers the organism occurs sparsely amongst other organisms, but in others in fairly large proportion, and occasionally in almost pure culture. In the great majority of carriers the organism can be found for only a comparatively short time—a few days even, or a week or two—but in a small proportion it persists for months, these being "chronic" carriers. Such individuals will, of course, act in maintaining the source of the infection, and it appears that the occurrence of the epidemic disease depends upon a dissemination of the organism through the community as evidenced by a high carrier rate, though the conditions which lead to the dissemination are not understood. Unfortunately we have at present no ready means of estimating the relative virulence of meningococci obtained from the naso-pharynx and from elsewhere. With regard to the epidemiology two facts are of importance. One is that direct infection of a healthy individual from a patient suffering from the disease is comparatively uncommon, though it sometimes occurs; the other is that it is rare for a known carrier to develop the disease. On the other hand, there is substantial evidence of persons being infected from carriers. The facts mentioned would seem to show that the organism is spread widely from individual to individual, in most cases without result, but that when the organism reaches a susceptible individual the disease may rapidly develop. No doubt the number of the organisms in the naso-pharynx is a factor of importance, heavy carriers being especially dangerous. It has been stated by some observers that the presence of the meningococcus leads to, or is associated with, pharyngeal catarrh, and that this often precedes meningeal infection. More extended observations, however, have thrown doubt on this, as it is certainly the case at least that the organism may abound in the naso-pharynx without the presence of catarrh or any abnormality. Manifestly the act of coughing, however, will aid in its diffusion when it is present.

Apart from the epidemic form of the disease, cases of a sporadic nature also occur, in which the lesions are of the same nature,

and in which the meningococcus is present. The facts stated would indicate that the origin and spread of the disease in the epidemic form depend on certain unknown conditions which produce an increased virulence of the organism. In *simple posterior basal meningitis* in children a diplococcus is present, as described by Still, which has the same microscopic and cultural characters as the meningococcus; it has been regarded as probably an attenuated variety of the latter. Houston and Rankin have found that the serum of a patient suffering from epidemic meningitis does not exert the same opsonic and agglutinative effects on the diplococcus of basal meningitis as on the meningococcus; and this result points to the two organisms being distinct, though closely allied, species.

Identification of the Meningococcus.—In the case of meningitis, this usually presents no difficulty, as the finding of a Gram-negative diplococcus in the cerebro-spinal fluid is practically conclusive. In the case of the naso-pharynx, however, the matter is quite different. Means must be taken to distinguish the organism from others resembling it, which occur in the situation. Till recently, the following points taken together have been usually accepted as justifying a positive diagnosis: conformity in the microscopic characters and in the appearance of the colonies with those of the meningococcus, ready emulsification in saline, absence of growth on agar at 23° C., fermentation of glucose and maltose, and non-fermentation of saccharose. Attempts have been made to obtain identification by means of agglutination. Dopter was the first to show that organisms obtained from the naso-pharynx, in other respects conforming to meningococci, might not be agglutinated by the serum of an animal immunised against the meningococcus; he applied the term *parameningococci* to such organisms. During the war Gordon did important work on this subject. On examining meningococci from various cases of meningitis, he found that a serum prepared by injecting any one strain did not agglutinate all the strains separated. Proceeding further and preparing sera for other strains which were not agglutinated, he arrived finally at the recognition of four "types" (I.–IV.), according to agglutinating tests, cross agglutination between them being little marked. Of these, Types I. and II. are the commonest, the latter being rather the more frequent. All the strains separated from cases of meningitis were found to be agglutinated by one of the four sera. The inference is that diplococci otherwise like meningococci, which are not agglutinated by any of the type sera, are without pathogenic significance, and are not accepted as true meningococci. Gordon's results in military cases proved of much practical value, but they have been criticised by Griffith and Scott and by Fildes, who point out that not all meningococci are agglutinated by one of Gordon's monovalent type sera, and that accordingly some meningococci in the naso-pharynx may escape detection by this method of testing. Fildes also states that while a rabbit tends to produce a monovalent serum on being inoculated with a strain of meningococcus, the horse when

similarly inoculated may produce a strain which is polyvalent. On testing an anti-meningococcic serum from the horse he found that it agglutinated all the meningococci tested. At present it appears hardly justifiable to conclude that an organism may not be the meningococcus merely because it is not agglutinated by certain sera, provided that it conforms in other respects.

Preparation of Agglutinating Sera.—Hine has devised the following method in the case of meningococci. A rabbit receives on one day three intravenous injections of 500 millions of dead meningococci, with an interval of an hour between the injections; six days afterwards it receives a single dose of 3000 millions. On the eighth day the serum has usually a titre of over 1 : 800. Young rabbits of about a kilogramme in weight give the best results For testing, four dilutions are used, namely: 1 . 50, 1 : 100, 1 : 200, 1 : 400. Emulsions of known type organisms are used as controls at the same time After the mixtures are made they are put in a chamber at 55° C for twenty-four hours, and the results are then read

Serum Reactions.—An agglutination reaction towards the meningococcus is given by the serum of patients suffering from the disease, when life is prolonged for a sufficient length of time. It usually appears about the fourth day, when the serum may give a positive reaction in a dilution of 1 : 50; at a later stage it has been observed in so great a dilution as 1 : 1000. Specific opsonins may appear in the blood about the same time, and though they are not always proportional in amount to the agglutinins, the two classes of substances have pretty much the same significance, and may occasionally be of use in diagnosis when lumbar puncture fails to give positive results Although their presence in large amounts may be said to indicate a marked reaction, they do not supply information of much value in relation to prognosis. Immune-bodies, as shown by bactericidal and fixation of complement tests (pp. 129, 134), may also be developed in considerable amount in the course of the disease.

Antisera for therapeutical purposes have been introduced by various workers, and of these the one which has been most extensively used is that of Flexner and Jobling. The serum is prepared from the horse by repeated injections in increasing doses of dead cultures, followed by injections of culture autolysate and of living cultures, these two latter being best administered by the subcutaneous method. Several strains of meningococci are mixed together for purposes of injection, and the immunisation is continued over a period of several months. For treatment of the disease the serum is injected within the spinal dura, 30 c.c. being generally used for an injection in an adult, this being repeated on subsequent days. Some of the spinal fluid is removed, and then the serum is injected, undue pressure being avoided. This serum has been used on a large scale in various parts of the world, and there is general agreement as to its favourable effects—the mortality of the disease,

which is generally 70 to 80 per cent., having been reduced to about 30 per cent. or even less. By means of its use the tendency to the occurrence of chronic lesions has also been markedly diminished. The action of such antisera cannot as yet be fully explained. They certainly contain opsonins, agglutinins, immune-bodies which fix complement, and possibly also anti-endotoxins. After the injection the number of meningococci becomes markedly reduced, probably as a result of increased phagocytosis; there can scarcely be any direct bactericidal action owing to the absence of complement. During the war monovalent sera against each of the four types of meningococcus (p. 293) and also a polyvalent serum were prepared for military cases by Gordon and his co-workers. The standardisation of such antisera is a matter of some difficulty; at first the fixation of complement method was used (p. 134), but later the opsonic index was regarded with more favour as an index of the potency of the serum. Gordon has pointed out the importance of estimating the anti-endotoxic action, and has described a method for this purpose.

Mackenzie and Martin treated cases by the intraspinal injection of the fresh serum of patients suffering from the disease or who have recovered from it, such serum being in many cases rich in immune-bodies for the meningococcus, and possessing a greatly increased bactericidal action as compared with normal serum. Though the number of cases treated by this method was not large, a distinctly favourable result was obtained.

Allied Diplococci.—In the naso-pharynx there occur other Gram-negative diplococci which morphologically have a close resemblance to the meningococcus. Many of these are chromogenic, *e.g.*, *M. catarrhalis flavus* (*Neisseria flava*), and can thus be readily distinguished; others differ in their fermentative actions. Of these latter the *Diplococcus* or *Micrococcus catarrhalis* (*Neisseria catarrhalis*) has the closest resemblance to the meningococcus. In addition to occurring in health this organism has also been found in large numbers in catarrhal conditions of the pharynx and respiratory passages. Its microscopic appearances are practically similar to those described above, and it also occurs within leucocytes. Its colonies on serum agar, though on the whole they tend to be rather more opaque, closely resemble those of the meningococcus. The organism usually grows on gelatin at 20° C. without liquefying the medium, and it has none of the fermentative properties described above as belonging to the meningococcus (p. 290).

The *Diplococcus pharyngis siccus* (*Neisseria sicca*) grows at room temperature, and its colonies are very tough and adhere to the surface of the medium; it can thus readily be distinguished from the meningococcus. It has marked fermentative properties, acting on glucose, maltose, saccharose, and lævulose. The *Diplococcus mucosus* has colonies of slimy consistence; it grows at room temperature, and it forms capsules, which can be demonstrated by the method of Hiss. The points of difference between the meningococcus and the gonococcus are given on p. 302. There are various other Gram-negative species of diplococci, which can be readily distinguished, and which have no pathogenic importance so far as is known. A Gram-positive diplococcus called the *Diplococcus crassus* is also of common occurrence; it is rather larger than the meningococcus, and, especially in subcultures, may tend to assume staphylococcal forms.

Meningitis due to other Organisms.—Meningitis may also be produced by almost any of the organisms described in the previous chapter, as associated with inflammatory conditions. A considerable number of cases, especially in children, are due to the *pneumococcus*. In many instances where no other lesions are present the extension is by the Eustachian tube to the middle ear. In other cases the path of infection is from some other lesion by means of the blood stream. This organism also infects the meninges not infrequently in lobar pneumonia, and in some cases with head symptoms we have found it present where there was merely a condition of congestion. Occasionally epidemics of meningitis have been due to the pneumococcus. Sporadic cases are also met with, and this organism comes next in order of frequency to the meningococcus as the cause of primary meningitis. The *pneumobacillus* also has been found in a few cases. Meningitis is not infrequently produced by *streptococci*, especially when middle-ear disease is present, less frequently by one of the staphylococci; occasionally more than one organism may be concerned. In meningitis following influenza the *influenza bacillus* has been found in a few instances, but sometimes the pneumococcus is the causal agent. Sporadic cases of meningitis occur associated with organisms which resemble the influenza bacillus morphologically and also in presenting hæmophilic culture reactions, but which possess pathogenic properties for rabbits and guinea-pigs. Both in the cerebro-spinal fluid and in cultures, these bacilli frequently show a tendency to produce long filamentous forms and also may show a beading of the protoplasm, which gives them a diph-

theroid appearance (*vide* p. 491). The cases from which such bacilli have been isolated have chiefly occurred in children, are extremely fatal, and probably often follow on an otitis media, from which condition similar organisms have been isolated. Sometimes the meningitis is part of a septicæmic or pyæmic process—in the latter the joints are often affected. It is impossible at present to say whether the organisms associated with such conditions are true influenza bacilli or are merely allied to them. They certainly tend to be more widely distributed in the body of the infected individual than is the case in the disease known clinically as influenza. On the other hand, influenza appears under several forms, and considerable variations may exist in the virulence of strains responsible for different outbreaks. An invasion of the meninges by the *anthrax bacillus* occurs, but is a rare condition; it is attended by diffuse hæmorrhage in the sub-arachnoid space. In tubercular meningitis the *tubercle bacillus*, of course, is present, especially in the nodules along the sheaths of the vessels.

In conclusion, it may be stated that *mixed infections* may occur in meningitis. Thus the pneumococcus has been found associated with the tubercle bacillus and also with the meningococcus, sometimes appearing as an additional infection to the latter.

Methods of Examination.—During life these involve the microscopic investigation of the centrifuged cerebro-spinal fluid and making cultures therefrom (p. 147). For the former, smears stained by methylene-blue and by Gram's method make the recognition of the meningococcus relatively easy, and the presence of Gram-negative cocci, especially within cells, is practically diagnostic of a case of cerebro-spinal fever. Tubes of trypagar, serum agar (pp. 54, 55), or boiled blood agar may then be inoculated. The difficult cases are those where no bacteria can be found microscopically in the lumbar fluid. Here the character of the exudate may give help. A predominance of polymorpho-nuclear cells is usually manifest in meningococcic, pneumococcic, and influenzal cases, whereas in tubercular meningitis the exudate is, as a rule, chiefly lymphocytic, though polymorphs, often degenerated, also occur. In such circumstances, besides other media, a tube of blood-smeared agar should be inoculated in case the pneumococcus or the influenza bacillus is the causal organism. Also several c.c. of the fluid mixed with an equal volume of 1 per cent. glucose broth should be incubated at 37° C., as by this means meningococci can sometimes be recovered when cultures from

the sediment remain sterile. To speak generally, if with a polymorpho-nuclear exudate no growth occurs in the media mentioned, the case is most likely to be due to the meningococcus. The isolation of the organism from the naso-pharynx will give confirmatory, though of course not conclusive, evidence. It must be kept in view, however, that in meningitis high up, produced by any of the organisms mentioned, polymorpho-nuclear leucocytes may be present in the fluid obtained by lumbar puncture before the organisms themselves appear. In tubercular cases it is sometimes impossible to demonstrate the bacilli microscopically in the exudate, though on careful search they may usually be found.

For method of examination of the naso-pharynx, *vide* p. 148.

CHAPTER IX

GONORRHŒA AND SOFT SORE

Gonorrhœa

Introductory.—The micrococcus now known to be the cause of gonorrhœa, and called the *Gonococcus*, was first described by Neisser, who in 1879 gave an account of its microscopical characters as seen in the pus of gonorrhœal affections, both of the urethra and of the conjunctiva. He considered that this organism was peculiar to the disease, and that its characters were distinctive. Later it was successfully isolated and cultivated on solidified human serum by Bumm and others. Its characters have since been minutely studied, and by inoculation of cultures in the human subject its causal relationship to the disease has been conclusively established.

The Gonococcus (*Neisseria gonorrhœæ*).—**Microscopical Characters.**—The organism of gonorrhœa is a micrococcus which usually is seen in diplococcal form, the adjacent margins of the two cocci being flattened, or even slightly concave, so that between them there is a small oval unstained interval. An appearance is thus presented which has been compared to that of two beans placed side by side (*vide* Fig. 68). When division takes place in the two members of a diplococcus, a tetrad is formed, which, however, soon separates into two sets of diplococci —that is to say, arrangement as diplococci is much commoner than as tetrads. Cocci in process of degeneration are seen as spherical elements of varying size, some being considerably swollen and staining faintly.

These organisms are found in large numbers in the pus of acute gonorrhœa, both in the male and female, and for the most part are contained within the leucocytes. In the earliest stage, when the secretion is glairy, a considerable number are lying free, or are adhering to the surface of desquamated epithelial cells, but when it becomes purulent the large proportion within leucocytes is a very striking feature. In the leucocytes they lie within the protoplasm, especially superficially, and are often so

numerous that the leucocytes appear to be filled with them, and their nuclei are obscured. It has been observed that the marked and characteristic phagocytosis of the organism only occurs in the purulent exudate and not in the inflamed tissue. As the disease becomes more chronic, the gonococci gradually become fewer, though even in long-standing cases they may still be found in considerable numbers. They are also present in the purulent secretion of gonorrhœal conjunctivitis, in various parts of the female genital organs when these are the seat of gonorrhœal infection, and they have been found in some cases in the secondary infections of the joints, as will be described below.

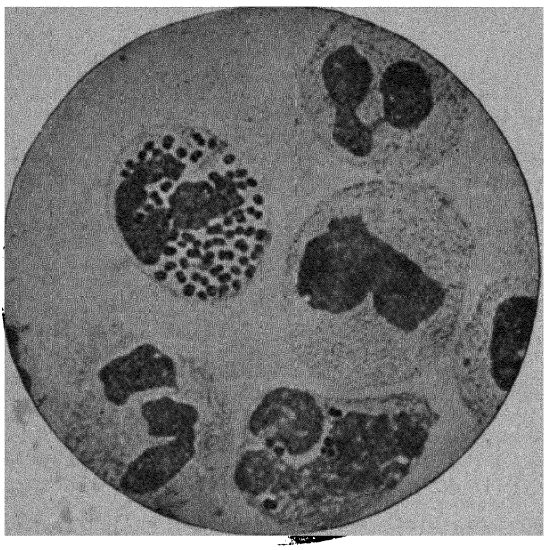


FIG. 68.—Portion of film of gonorrhœal pus, showing the characteristic arrangement of the gonococci within leucocytes. See also Plate I., Fig. 5. Stained with fuchsin. ×1000.

Staining.—The gonococcus stains readily and deeply with a watery solution of any of the basic aniline dyes—methylene-blue, fuchsin, etc. It is, however, easily decolorised, and is Gram-negative—an important point in microscopical diagnosis.

Cultivation of the Gonococcus.—This is attended with some difficulty, as the conditions of growth are somewhat restricted. Blood or serum, incorporated in the culture medium, is necessary, and a variety of media have been advocated by different workers for the cultivation of the organism. The most suitable for routine culture are "blood agar" and the serum media[1] already described for the purpose (p. 54). The optimum P_H is from 6·8 to 7·4 (Torrey and Buckell). It is advisable to inoculate the media within half an hour after obtaining the material from the body, and to place the tubes at once in the incubator. Growth takes place best at the temperature of the body, and ceases altogether at 25° C. Cultures are obtained by taking some pus on the loop of the platinum needle and inoculating plates of the medium by successive strokes. The colonies are usually visible within forty-eight hours, and

[1] Certain media, too complicated to give in detail, have been introduced with much success, *e.g.* by Tulloch (*Journ. Path. and Bact.*, 1922, xxv. 346).

often within twenty-four hours ; it is important, however, to note that sometimes growth may not appear till the fourth day.

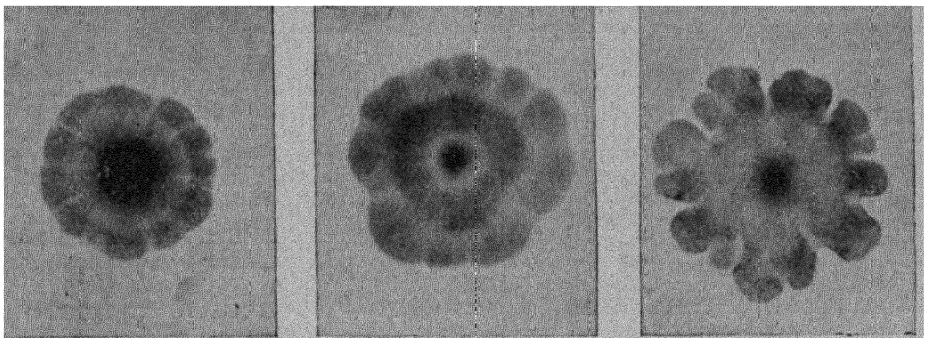


FIG. 69.—Colonies of gonococcus on serum agar ; (*a*) three days' growth ; (*b*) and (*c*) five days' growth. ×9.
From photographs by the late Prof. W. B. M. Martin.

Different strains seem to vary in their ability to grow on artificial media in primary culture. The colonies are small, semi-transparent, rounded discs, and vary somewhat in size. They tend to remain more or less separate. Later, the margin may become undulated and the centre more opaque ; radial and concentric markings may be present (Fig. 69). The first cultures die out somewhat quickly, but in subcultures, kept at 37° C., the organism remains alive for a considerable time, sometimes three weeks. After about a week, more active foci of growth may appear in some of the colonies in the form of papillæ. Atkin has recently drawn attention to two types of colonies observed on trypagar, which apparently represent different biological types of the organism : (I.) large, irregular, thin colonies with papillæ ; (II.) smaller, rounder, thicker, and denser colonies

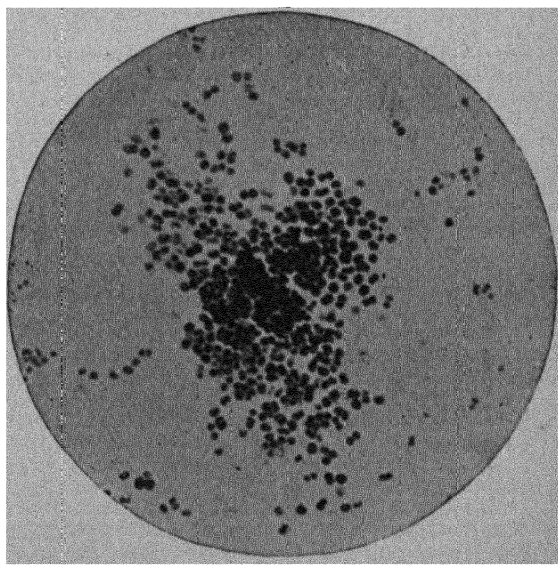


FIG. 70.—Gonococci, from a pure culture on blood agar of twenty-four hours' growth. Some cocci already are beginning to show the swollen appearance common in older cultures.
Stained with carbol-thionin-blue. ×1000.

without papillæ. He claims that the papillæ of type I. are more viable than their substrate, and that type II. colonies are derived from these papillæ of type I.

In the early stage of the disease the organism is present in the male urethra in practically pure condition, and if the meatus of the urethra be sterilised by washing with a weak solution of corrosive sublimate and then with absolute alcohol, and the material for inoculation be expressed from the deeper part of the urethra, cultures may often be obtained which are practically pure from the first. In culture, the organisms have similar microscopic characters to those described (Fig. 70), but show a remarkable tendency to undergo degeneration, becoming swollen and of various sizes, and staining very irregularly. Degenerated forms are seen even on the second day, whilst in a culture four or five days old comparatively few normal cocci may be found. The less suitable the medium the more rapidly does degeneration take place. When mixed with other organisms the gonococcus may be separated by plate cultures on the media mentioned in the usual way.

Comparison with Meningococcus.—The morphological and cultural characters of the gonococcus and meningococcus are in many respects closely similar; the following points are of importance in distinguishing them biologically. The conditions of growth of the gonococcus are more restricted than those of the meningococcus. The gonococcus does not grow on the ordinary agar media, whereas the meningococcus may grow slightly after repeated subculture. The colonies of the latter are generally more opaque and have more regular margins than those of the gonococcus. In fermentative reactions glucose is the only sugar usually employed which is fermented by the gonococcus, whereas the meningococcus ferments maltose also. (For fermentative tests in the case of the gonococcus, solid media should be used, sloped agar containing 5 per cent. sterile unheated serum, with litmus and the particular sugar added, being specially suitable.)

Serological Types of the Gonococcus.—A study of the immunity reactions of the gonococcus with antisera has elicited the fact that this species of organism is serologically heterogeneous. The agglutination and agglutinin-absorption reactions have been mostly studied, and the general result of different workers has been that gonococcus strains can be classified into a number of serologically distinct types. Torrey and Buckell find that gonococcus strains cannot be classified into such well-defined serological types as in the case of the pneumococcus (*q.v.*), and that certain strains possess generalised antigenic characters common to the species as a whole. They have also observed variations in individual strains at different times as regards their antigenic properties. According to Tulloch (*op. cit.* p. 300), 72 per cent. of cases of acute and subacute gonorrhœa in the male are caused by one fairly well defined serological type.

Relations to the Disease.—The gonococcus is invariably present in the urethral discharge in gonorrhœa, and also in other parts of the genital tract when these are the seat of gonorrhœal infection. Its presence in these different positions has been demonstrated not only by microscopical examination but also by culture. From the description of the conditions of growth in culture it will be seen that life outside the body in natural conditions is practically impossible—a statement which corresponds with the clinical fact that the disease is always transmitted directly by contagion. Inoculation of pure cultures in the urethra of lower animals, and even of apes, is followed by no effect, but a similar statement can be made with regard to inoculation of gonorrhœal pus itself. In fact, hitherto it has been found impossible to reproduce the disease by any means in the lower animals. On a considerable number of occasions inoculations of pure cultures have been made in the human urethra, both in the case of the male and female, and the disease, with all its characteristic symptoms, has resulted. (Such experiments have been performed independently by Bumm, Steinschneider, Wertheim, and others.) The causal relationship of the organism to the disease has therefore been completely established.

Intraperitoneal injections of pure cultures of the gonococcus in white mice produce a localised peritonitis with a small amount of suppuration, the organisms being found in large numbers in the leucocytes (Wertheim). They also penetrate the peritoneal lining and are found in the sub-endothelial connective tissue, but they appear to have little power of proliferation, they soon disappear, and the inflammatory condition does not spread. Injection of pure cultures into the joints of rabbits, dogs, and guinea-pigs causes an acute inflammation, which, however, soon subsides, whilst the gonococci rapidly die out; a practically similar result is obtained when dead cultures are used. These experiments show that while the organism, when present in large numbers, can produce a certain amount of inflammatory change in these animals, it has little or no power of multiplying and spreading in their tissues.

Toxin of the Gonococcus.—De Christmas cultivated the gonococcus in a mixture of 1 part of ascitic fluid and 3 parts of bouillon, and found that the fluid after twelve days' growth had toxic properties. At this period all the organisms were dead, and the fluid constituted the "toxin." The toxic substances were precipitated along with the proteins by alcohol, and the precipitate after being desiccated possessed the toxic action. In young rabbits injection of the toxin produced suppuration; this was well seen in the anterior chamber of the eye, where hypopyon resulted. The most interesting point, however, was with regard to its action on mucous surfaces; for, while in the case of animals it produced no effect, its introduction into the human urethra caused acute catarrh,

attended with purulent discharge. He found that no tolerance to the toxin resulted after five successive injections at intervals. In a later publication he pointed out that the toxin on intra-cerebral injection had marked effects ; he also claimed to have produced an antitoxin. The production of a soluble toxin as described by De Christmas has not been confirmed by subsequent workers.

Distribution in the Tissues.—The gonococcus having been thus shown to be the direct cause of the disease, some additional facts may be given regarding its presence both in the primary and secondary lesions. In the human urethra the gonococci penetrate the mucous membrane, passing chiefly between the epithelial cells, causing a loosening and desquamation of many of the latter and inflammatory reaction in the tissues below, attended with great increase of secretion. There occurs also a gradually increasing emigration of leucocytes, which take up a large number of the organisms. The organisms also penetrate the subjacent connective tissue and are especially found, associated with extensive leucocytic emigration, around the lacunæ. Even, however, when the gonococci have disappeared from the urethral discharge, they may still be present in the deeper part of the mucous membrane of the urethra, and also in the prostate, and may thus be capable of producing infection. The prostatic secretion may be examined by making pressure on the prostate from the rectum (" prostatic massage ") when the patient has almost emptied his bladder, the secretion being afterwards discharged along with the remaining urine. In acute gonorrhœa there is often a considerable degree of inflammatory affection of the prostate and vesiculæ seminales, but it is difficult to say whether these conditions are always due to the presence of gonococci in the affected parts. A similar statement also applies to the occurrence of orchitis and also of cystitis in the early stage of gonorrhœa. Gonococci have, however, been obtained in pure culture from peri-urethral abscess and from epididymitis : it is likely that the latter condition, when occurring in gonorrhœa, is usually due to the actual presence of gonococci. During the more chronic stages other organisms may appear in the urethra, aid in maintaining the irritation, and may produce some of the secondary results. The pyogenic cocci, the B. coli, diphtheroid bacilli, etc., are often present, and may extend along the urethra to the bladder and set up cystitis, though in this they may be aided by the passage of a catheter. It may be mentioned here that Wertheim cultivated the gonococcus from a case of chronic gonorrhœa of two years' standing, and by inoculation in the human subject proved it to be still virulent.

In the disease in the female, gonococcci are almost invariably present in the urethra, the situation affected next in frequency being the cervix uteri. They do not appear to infect the lining epithelium of the vagina of the adult unless some other abnormal condition be present, but they do so in the gonorrhœal vulvo-vaginitis of young subjects. They have also been found in suppurations in connection with Bartholini's glands, and sometimes produce an inflammatory condition of the mucous membrane of the body of the uterus. They may also pass along the Fallopian tubes and produce inflammation of the mucous membrane there. From the pus in cases of pyosalpinx they have been cultivated in a considerable number of cases. According to the results of various observers they have been found in one out of four or five cases of this condition, usually unassociated with other organisms. Further, in a large proportion of the cases in which the gonococcus has not been found, no organisms of any kind have been obtained from the pus, and in these cases the gonococci may have been once present and have subsequently died out. Lastly, they may pass to the peritoneum and produce peritonitis, which is usually of a local character.

In *gonorrhœal conjunctivitis* the mode in which the gonococci spread through the epithelium to the subjacent connective tissue is similar to what obtains in the case of the urethra. Their relation to the leucocytes in the purulent secretion is also the same. Microscopic examination of the secretion alone in acute cases often gives positive evidence, and pure cultures may be readily obtained. As the condition becomes more chronic, gonococci are less numerous and a greater proportion of other organisms may be present. An infection of the conjunctiva in new-born children, *ophthalmia neonatorum*, which is often of very severe nature, is also due to the gonococcus.

Relations to Joint-Affections, etc.—The relations of the gonococcus to the sequelæ of gonorrhœa form a subject of great interest and importance, and the application of recent methods of examination shows that the organism is much more frequently present in such conditions than the earlier results indicated. The following statements may be made with regard to them: First, in a large number of cases of arthritis following gonorrhœa pure cultures of the gonococcus may be obtained. A similar statement applies to inflammation of the sheaths of tendons following gonorrhœa. Secondly, in a considerable proportion of cases no organisms have been found. It is, however, probable that in many of these the gonococci may have been present in the synovial membrane, as it has been observed that they may

be much more numerous in that situation than in the fluid. Thirdly, in some cases, especially in those associated with extensive suppuration, occasionally of a pyæmic nature, various pyogenic cocci have been found to be present. In the instances in which the gonococcus has been found in the joints, the fluid present has usually been described as being of a whitish-yellow tint, somewhat turbid, and containing shreds of fibrin-like material, though sometimes purulent in appearance. In one case Bordoni-Uffreduzzi cultivated the gonococcus from a joint-affection, and afterwards produced gonorrhœa in the human subject by inoculating with the cultures obtained. In another case, in which pleurisy was present along with arthritis, the gonococcus was cultivated from the fluid in the pleural cavity. The existence of a *gonorrhœal endocarditis* has also been established. Cases apparently of this nature occurring in the course of gonorrhœa had been previously described, but the complete bacteriological test has been satisfied in several instances. In one case Lenhartz produced gonorrhœa in the human subject by inoculation with the organisms obtained from the vegetations. A true *gonorrhœal septicæmia* has also been demonstrated, cultures of the gonococcus having been obtained from the blood during life on more than one occasion (Thayer and Blumer, Thayer and Lazear, Ahmann). Such occurrences are, however, rare.

Immunity and Serum Diagnosis.—Immunity following recovery from the infection is usually transient. Specific antibodies have been demonstrated in the serum of persons infected with the gonococcus, *e g.* agglutinin, and immune-body; and the complement-fixation test has now been extensively applied in the diagnosis of gonorrhœal infections in which the organism cannot be demonstrated by direct methods. The general principles and technique of the method are those described in Chapter IV. For the test a polyvalent antigen must be used, consisting of a number of strains sufficient to represent the different serological races of the gonococcus. Early cases rarely exhibit a positive reaction, and a negative result may be of no diagnostic significance. Repeated negative reactions after treatment have been regarded as indicative of cure. The test is of only limited application and value, and reactions when they occur are often comparatively weak.

Vaccines.—Both gonorrhœa itself and the secondary infections have been treated by means of vaccines, but the results reported vary greatly. On the whole, most success has been obtained in the case of joint infections and allied conditions, though even here reports are contradictory. The initial dose employed has been usually about five million cocci, but care is necessary in starting the treatment, especially in the case of acute gonorrhœa.

Serum Therapy.—Specific antisera have been applied in the treatment of gonorrhœa.

Methods of Diagnosis.—For microscopical examination, dried films of the suspected pus, etc., may be stained by any of the simple solutions of the basic aniline stains. Methylene- or thionin-blue may be used. Staining for one minute is sufficient. It is also necessary to stain by Gram's method, and it is advisable to put alongside the pus film a small quantity of culture of staphylococcus as a " control spot " in staining by this method ; the control should stain deeply violet, while the gonococci are coloured only with the counter-stain. Regarding the value of microscopic examination alone, we may say that the presence in a urethral discharge of a large number of diplococci having the characters, position, and staining reactions described above, is practically conclusive that the case is one of gonorrhœa. There is no other condition in which this sum-total of microscopical characters is present. A similar statement applies to conjunctivitis. Even in chronic cases the typical picture is often well maintained, and microscopic examination alone may give a definitely positive result. When other organisms are present, and especially when the gonococci are few in number, it is difficult, and in some cases impossible, to give a definite opinion, as a few gonococci mixed with other organisms cannot be recognised with certainty. This is often the condition in chronic gonorrhœa in the female In the case of the female a drop of secretion should be taken on a platinum loop from the urethra or, with the aid of a speculum, from the cervix uteri, the adjacent parts being cleansed as far as possible by swabbing with sterile cotton wool. Microscopic examination, therefore, though often giving positive results, will sometimes be inconclusive In such cases cultures in suitable media (*vide supra*) should be made if possible, though in a mixed infection it may be difficult to isolate and identify the organism. As regards lesions in other parts of the body, microscopic examination alone is quite insufficient ; it is impossible, for example, to distinguish by this means the gonococcus from other Gram-negative diplococci. Cultures alone supply the test, and the points above detailed are to be attended to

The complement-fixation test (*vide supra*) may be applied in suspected cases in which gonococci cannot be demonstrated by direct methods.

Soft Sore (Chancroid)

The bacillus of soft sore (*Hæmophilus ducreyii*) was first described by Ducrey in 1889, who found it in the purulent discharge from the ulcerated surface ; and later, in 1892, Unna described its appearance and distribution as seen in sections through the sores. The statements of these observers regarding the presence and characters of this organism have been fully confirmed by other observers.

Microscopical Characters.—The organism occurs in the form of minute oval rods measuring about 1·5 μ in length, and 0·5 μ in thickness (Fig. 71). It is found mixed with other organisms in the purulent discharge from the surface, and is chiefly arranged

in small groups or in short chains. When studied in sections through the ulcer, it is found in the superficial part of the floor, but more deeply situated than other organisms, and may be present in a state of purity in the leucocytic infiltration. In this position it is usually arranged in chains, which may be of considerable length, and which are often seen lying in parallel rows between the cells. The bacilli chiefly occur in the free condition, but occasionally a few may be contained within leucocytes.

There is no doubt that in many cases the organism is present in the buboes in a state of purity; it has been found there by microscopic examination, and cultures have also been obtained from this source. The negative results of some observers are probably due to the organism having died off. On the whole, the evidence goes to show that the bubo associated with soft sore is to be regarded as a secondary lesion produced by Ducrey's bacillus. Sometimes the ordinary pyogenic organisms become superadded.

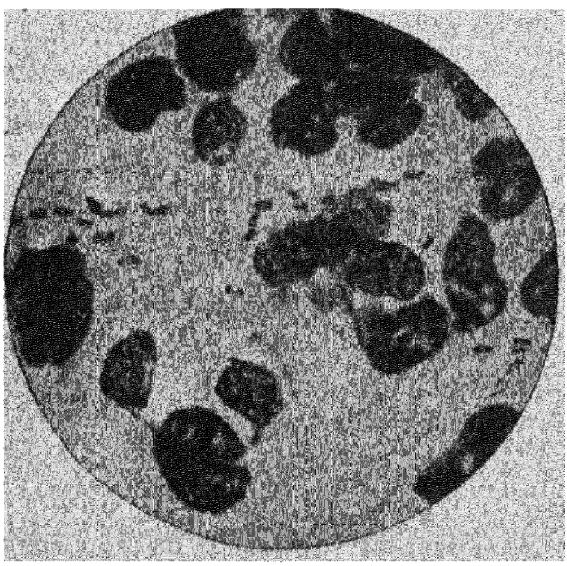


FIG. 71.—Film preparation of pus from soft chancre, showing Ducrey's bacillus, chiefly arranged in pairs. Stained with carbol-fuchsin and slightly decolorised. ×1500.

This bacillus takes up the basic aniline stains fairly readily, but loses the colour very rapidly when a decolorising agent is applied. Accordingly, in film preparations when dehydration is not required, it can be readily stained by most of the ordinary combinations, though Löffler's methylene-blue solution is preferable, as it does not over-stain. In sections, however, great care must be taken in the process of dehydration, and the aniline-oil method (*vide* p. 101) should be used for this purpose, as alcohol decolorises the organism very readily. Ducrey's bacillus stains negatively by Gram's method.

Cultivation.—For a long period of time attempts to obtain cultures were unsuccessful. Benzançon, Griffon, and Le Sourd obtained pure cultures in four cases, the medium used being a mixture of rabbit's blood and agar, in the proportion of 1

part of the former to 2 of the latter. The blood was added to the agar in the melted condition at 45° C., and the tubes were then sloped. Davis confirmed these results, and found that another good medium is freshly drawn human blood distributed in small tubes; this method is specially suitable, as the blood inhibits the growth of various extraneous organisms. Initial cultures from sores can also be readily obtained in tubes containing 1 or 2 c.c. of sterile rabbit's blood which has been allowed to coagulate and has then been heated at 55° C. The primary growth obtained in this way is subcultured on blood agar. Cultures may also be made by this method from the buboes, the inoculum being obtained by puncturing the enlarged gland with a syringe. On solid medium the growth appears in the form of small round globules, which attain their complete development in forty-eight hours, having then a diameter of 1 to 2 mm.; the colonies do not become confluent. Microscopic examination of these colonies, which are dissociated with some difficulty, shows appearances similar to those observed when the organism is in the tissues (Fig. 72), but occasionally long undivided filaments are observed. Within a comparatively short period cultures undergo marked degenerative changes, and great irregularities of form and shape are to be found. It would appear that a comparatively large amount of blood is necessary for the growth of this organism, and even subcultures on the ordinary media, including blood-serum media, give negative results. Inoculation of the ordinary laboratory animals is not attended by any result, but it has been found that some monkeys are susceptible, small ulcerations being produced by superficial inoculation, and in all these the organism can be demonstrated. Tomasczewski cultivated the organism for several generations, and reproduced

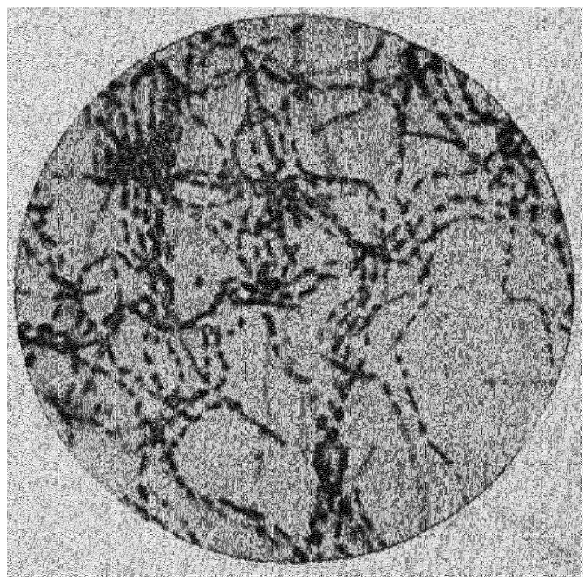


FIG. 72.—Ducrey's bacillus from a twenty four-hour culture in blood-bouillon. ×1500.[1]

[1] We are indebted to Dr. Davis for the use of Figs. 71 and 72.

the disease by inoculation of the human subject. The causal relationship of this bacillus must therefore be considered as completely established, and the conditions under which it grows show it to be a strict parasite under natural conditions—a fact which is in conformity with the known facts as to the transmission of the disease.

CHAPTER X

TUBERCULOSIS

THE cause of tuberculosis was proved by Koch in 1882 to be the organism now known as the tubercle bacillus. Probably no other single discovery has had a more important effect on medical science and pathology than this. It has supplied infallible methods for determining the tubercular nature of lesions, and has also given the means of studying the modes and paths of infection.

Historical.—By the work of Armanni and of Cohnheim and Salomonsen (1870–80) it had been demonstrated that tubercle was an infective disease. The latter observers found on inoculation of the anterior chamber of the eye of rabbits with tubercular material, that in many cases the results of irritation soon disappeared, but that after a period of incubation, usually about twenty-five days, small tubercular nodules appeared in the iris ; afterwards the disease gradually spread, leading to disorganisation of the globe of the eye. Later still, the lymphatic glands became involved, and finally the animal died of acute tuberculosis. The question remained as to the nature of the virus, the specific character of which was thus established, and this question was answered by the work of Koch, which will remain as a classical masterpiece of bacteriological research, both on account of the great difficulties which he successfully overcame and the completeness with which he demonstrated the relations of the organism to the disease. The two chief difficulties were, first, the demonstration of the bacilli in the tissues, and, secondly, the cultivation of the organism outside the body. For, with regard to the first, the tubercle bacillus cannot be demonstrated by a simple watery solution of a basic aniline dye, and it was only after staining for twenty-four hours, with a solution of methylene-blue with caustic potash added, that he was able to reveal the presence of the organism. Then, in the second place, all attempts to cultivate it on the ordinary media failed, and he succeeded in obtaining growth only on solidified blood serum, the method of preparing which he himself devised, inoculations being made on this medium from the organs of animals artificially rendered tubercular. The fact that growth did not appear till the tenth day at the earliest, might easily have led to the hasty conclusion that no growth took place. The organism was cultivated by the above method from a great variety of sources, and by a large series of inoculation experiments on various animals, performed by different

methods, Koch conclusively proved that bacilli from these different sources produced the same tubercular lesions and were really of the same species. His work demonstrated that such conditions as lupus, "white swelling" of joints, scrofulous disease of glands, etc., are really tubercular in nature.

Tuberculosis in Animals.—Tuberculosis is not only the most widely spread of all infective diseases affecting the human subject, and is one of the chief causes of death, but there is probably no other disease which affects the domestic animals so widely. We need not here describe in detail the various tubercular lesions in the human subject, but some facts regarding the disease in the lower animals may be given, as this subject is of great importance in relation to the infection of the human subject.

Amongst the domestic animals the disease is commonest in cattle (bovine tuberculosis), in which animals the lesions are very various, both in character and distribution. In most cases the lungs are affected, and contain numerous rounded nodules, many being of considerable size; these may be softened in the centre, but are usually of pretty firm consistence and may be calcified. There may be in addition caseous pneumonia, and also small tubercular granulations. Along with these changes in the lungs, the pleuræ are also often affected, and show numerous nodules, some of which may be of large size, firm and pedunculated, the condition being known in Germany as *Perlsucht*, in France as *pommelière*. Lesions similar to the last may be chiefly confined to the peritoneum and pleuræ. In other cases, again, the abdominal organs are principally involved. The udder becomes affected in a certain proportion of cases of tuberculosis in cows—in 3 per cent. according to Bang—but primary affection of this gland is very rare. Tuberculosis is also a comparatively common disease in pigs, in which animals it in many cases affects the abdominal organs, in other cases produces a sort of caseous pneumonia, and sometimes is met with as a chronic disease of the lymphatic glands, the so-called "scrofula" of pigs. Tubercular lesions in the muscles are less rare in pigs than in most other animals. In the horse the abdominal organs are usually the primary seat of the disease, the spleen being often enormously enlarged and crowded with nodules; sometimes, however, the primary lesions are pulmonary. In sheep and goats tuberculosis is of rare occurrence, especially in the former animals. It may occur spontaneously in dogs, cats, and in the large carnivora. It is also sometimes met with in monkeys in confinement, and leads to a very rapid and widespread affection, the nodules having a special tendency to soften and break down into a pus-like fluid.

Tuberculosis in fowls (avian tuberculosis) is a common and very infectious disease, nearly all the birds in a poultry-yard being sometimes affected.

The disease in animals thus presents great variations in character, and may differ in many respects from that met with

in the human subject. The relations of the different forms of tuberculosis are discussed below, but it may be stated here that two chief types of mammalian tubercle bacilli are now recognised —a *human type* which is the common cause of tuberculosis in the human subject, and a *bovine type* which produces bovine tuberculosis and also a certain proportion of cases of human tuberculosis. The description which follows applies to the *human type*.

Tubercle Bacillus (Human Type) (*Mycobacterium tuberculosis hominis*)—**Microscopical Characters.**—Tubercle bacilli are minute rods which usually measure 2·5 to 3·5 μ in length, and 0·3 μ in thickness, *i.e.* in proportion to their length they are comparatively thin organisms (Figs. 73 and 74). Sometimes, however, longer forms, up to 5 μ or more in length, are met with, both in cultures and in the tissues. They are straight or slightly curved, and are of uniform thickness, or may show slight swelling at their extremities. When stained they appear uniformly coloured, or may present small uncoloured spots along their course, with darkly stained parts between. There is no satisfactory evidence that such appearances represent spore formation, and it has been shown that "beaded" bacilli have no higher powers of resistance than those which stain uniformly.



FIG. 73.—Tubercle bacilli of the human type, from a pure culture on glycerin agar.
Stained with carbol-fuchsin. ×1000.

The bacilli in the tissues occur scattered irregularly or in little masses. They are usually single, or two are attached end to end and often form in such a case an obtuse angle. True chains are not formed, but occasionally short filaments are met with. In cultures the bacilli form masses in which the rods are closely applied to one another and arranged in a more or less parallel manner. Tubercle bacilli are devoid of motility.

Aberrant Forms.—Though such are the characters of the organism as usually met with, other appearances are sometimes found. In old cultures, for example, very much larger elements may occur. These may be in the form of long filaments, sometimes swollen or

clubbed at their extremities, may be irregularly beaded, and may even show the appearance of branching. Their significance has been variously interpreted, for while some look upon them as degenerated or involution forms, others regard them as indicating a special phase in the life-history of the organism, allying it with the higher bacteria. The latter view is now generally accepted. It has also been found that tubercle bacilli in the tissues may produce radiating structures with club-like forms at the periphery closely similar to those of actinomyces. This was found by Babés and also by Lubarsch to be the case when the bacilli were injected under the

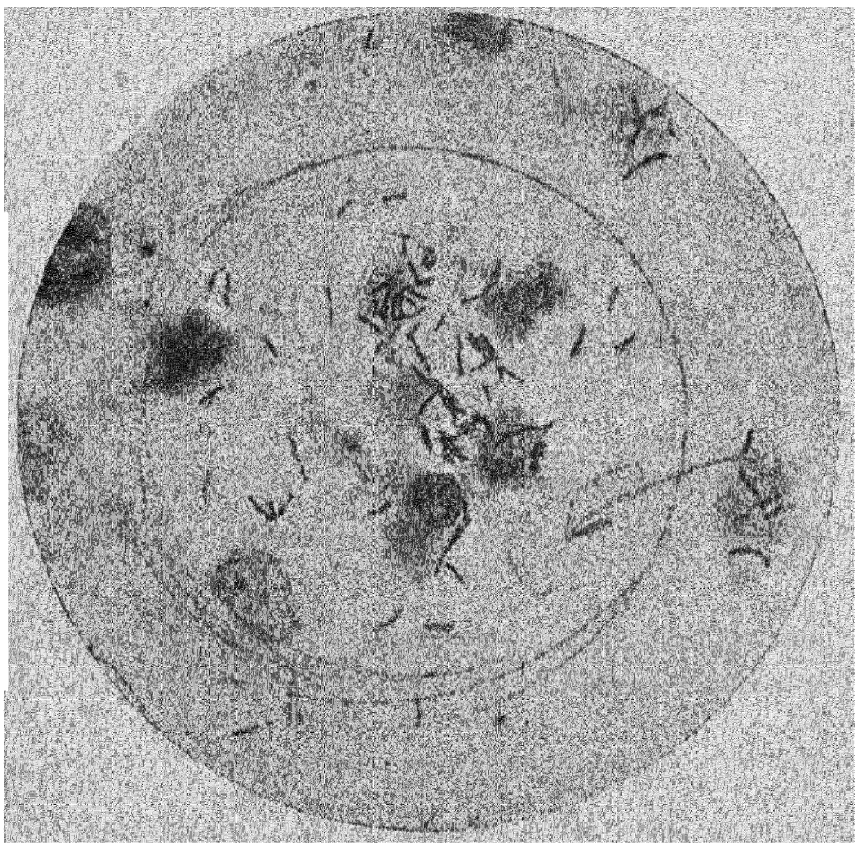


FIG. 74.—Tubercle bacilli in phthisical sputum ; they are longer than is often the case. See also Plate II., Fig. 7.
Film preparation, stained with carbol-fuchsin and methylene-blue.
×1000.

dura mater and directly into certain solid organs, such as the kidneys in the rabbit. Similar results obtained with other acid-fast bacilli will be mentioned below, and these organisms would appear to form a group closely allied to the streptothriceæ, the bacillary parasitic form being one stage of the life-history of the organism. This group is often spoken of as the *Mycobacteria*.

Staining Reactions.—The tubercle bacillus takes up the ordinary stains very slowly and faintly, and for successful staining one of the most powerful solutions ought to be employed,

e.g. gentian-violet or fuchsin, along with aniline-oil water or carbolic acid. Further, such staining solutions require to be applied for a long time, or the staining must be accelerated by heat, the solution being warmed till steam arises and the specimen allowed to remain in the hot stain for two or three minutes. One of the best and most convenient methods is the Ziehl-Neelsen method (see p. 109). The bacilli present this further peculiarity, discovered by Ehrlich, that after staining has taken place they resist decolorising by solutions which readily remove the colour from the tissues and from other organisms which may be present. Such decolorising agents are sulphuric or nitric acid in 20 per cent. solution. Preparations can thus be obtained in which the tubercle bacilli alone are coloured by the stain first used, and the tissues can then be coloured by a contrast stain. Other bacilli have been discovered which present the same staining reactions as tubercle bacilli; they are therefore called "acid-fast" (*vide infra*). The spores of many bacilli become decolorised more readily than tubercle bacilli, though some retain the colour with equal tenacity.

Much's Method.—Much maintained that the tubercle bacillus may exist in a form which is not acid-fast and also in the form of free granules. These two forms are demonstrable by certain modifications of Gram's method, of which the following is specially suitable:

Methyl-violet B.N., 10 c.c. of a saturated alcoholic solution in 100 c.c. of a 2 per cent. watery solution of carbolic acid; stain by boiling over the flame for a few minutes or at 37° C. for twenty-four to forty-eight hours, then treat with Gram's iodine for one to five minutes, 5 per cent. nitric acid for one minute, 3 per cent. hydrochloric acid for ten seconds, and complete the decolorisation with a mixture of acetone and alcohol in equal parts.

There seems to be no doubt that in certain conditions more tubercle bacilli can be demonstrated in the tissues by Much's method than by the Ziehl-Neelsen method. Sometimes tubercle bacilli can be stained by Gram's method employed in the usual way.

Chemical Composition.—When tubercle bacilli are extracted with organic solvents such as alcohol or ether, especially with the aid of heat, substances are dissolved out which show the acid-fast property. But it is extremely difficult to deprive the bacilli of their characteristic staining reaction by such treatment. Aronson found that extraction with a boiling mixture of hydrochloric acid and alcohol removed the acid-fast property, and Bulloch and M'Leod by a similar process extracted an acid-fast wax. Long has shown that if tubercle bacilli are extracted as completely as possible with alcohol and petroleum ether and are then treated with N/1 HCl a subsequent extraction with the organic solvents removes a further quantity of "firmly bound" lipin substances and destroys the acid-fastness and integrity of the bacilli, both of which had resisted the primary extraction. But B. subtilis contains practically as

much of the firmly bound lipins although, like other non-acid-fast organisms, its total content in substances soluble in organic solvents is much less than that of tubercle bacilli. Browning and Gulbransen have found that films of tubercle bacilli are rapidly deprived of acid-fastness by treatment with a mixture of alcohol and chloroform containing a minute amount of HCl, *e.g.* N/100, whereas in the absence of the acid several months' treatment leaves the bacilli still acid-fast.

The facts support Long's view that it is the manner of distribution of the waxy or lipin substance in the bodies of the bacilli, rather than its inherent acid-fast character, which confers the typical staining properties on tubercle bacilli. This accords also with Koch's observation that the bacilli when disintegrated by trituration cease to be acid-fast. Bacilli which have lost their acid-fast properties by the above methods are still stainable by basic aniline dyes, though they take the staining feebly. Laidlaw and Dudley have recently obtained glycogen from tubercle bacilli and also a carbohydrate complex of the nature of a gum. The latter gives specific precipitation with an immune serum.

Cultivation.—The medium first used by Koch was inspissated blood serum (*vide* p. 52). If inoculations are made on this medium with tubercular material free from other organisms, there appear in from ten to fourteen days minute points of growth of dull whitish colour, rather irregular, and slightly raised above the surface (it is advisable to plant on the medium an actual piece of the tubercular tissue and to fix it in a break of the surface of the serum). Koch compared the appearance of these to that of small dry scales. In such cultures the growths usually reach only a comparatively small size and remain separate, becoming confluent only when many occur close together. In subcultures, however, growth is more luxuriant and may come to form a dull wrinkled film of whitish colour, which may cover the greater part of the surface of the serum and at the bottom of the tube may grow over the surface of the condensation water on to the glass (Fig. 75, A). The growth is always of a dull appearance, and has a considerable degree of consistence, so that it is difficult to dissociate a portion thoroughly in a drop of water. In older cultures the growth may acquire a slightly brownish or buff colour. When the small colonies are examined under a low power of the microscope, they are seen to be extending at the periphery in the form of wavy or sinuous streaks which radiate outward, and which have been compared to the flourishes of a pen. The central part shows similar markings closely interwoven. These streaks are composed of masses of the bacilli arranged in a more or less parallel manner.

On Dorset's *egg medium* and especially on *glycerin egg medium* the organism grows well, producing an abundant wrinkled layer which has usually a yellowish, buff, or pinkish colour. These media, particularly when made up with digest broth, are specially suitable for direct cultivation from the tissues.

On *glycerin agar*, which was first introduced by Nocard and Roux as a medium for the culture of the tubercle bacillus, growth takes place in subcultures at an earlier date and progresses more rapidly than on serum, but this medium is not suitable for obtaining cultures from the tissues, inoculations with tubercular material usually yielding a negative result. The growth has practically the same characters as on serum. The organism also flourishes well on *glycerin potato*, and this medium is suitable for primary cultures from tubercular lesions. In *glycerin broth*, especially when the layer is not deep, tubercle bacilli grow readily in the form of little white masses, which fall to the bottom and form a powdery layer. If, however, the growth be started on the surface, it spreads superficially as a dull whitish wrinkled pellicle which may reach the walls of the flask; this mode of growth is specially suitable for the production of tuberculin (*vide infra*). The culture has a peculiar fruity and not unpleasant odour. On ordinary agar and on gelatin media no growth takes place. The use of animal tissues in glycerin bouillon as a medium for the growth of the tubercle bacillus was introduced by Frugoni, and is one which gives excellent results. He recommends that small wedges of rabbit's

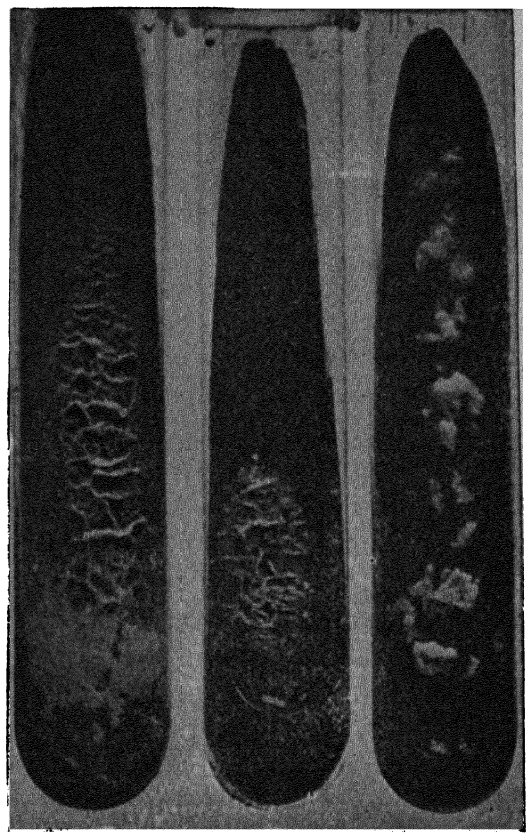


FIG. 75.—Cultures of tubercle bacilli on glycerin agar.

A and B. Mammalian tubercle bacilli of human type: A is an old culture, B one of a few weeks' growth.
C. Avian tubercle bacilli. The growth is whiter and smoother on the surface than the others.

lung should be sterilised in the autoclave, and placed in tubes of glycerin bouillon in such a way that their surface is kept moist by the medium, without the fragments being submerged. The growth is probably more rapid and luxuriant than in any other method.

The optimum temperature for growth is 37° to 38° C. Growth ceases about 42° and usually below 28°, but on long-continued cultivation outside the body and in special circumstances growth may take place at a lower temperature, *e.g.* Sander found that growth took place in glycerin-potato broth even at 22° to 23° C.

Powers of Resistance.—Tubercle bacilli have considerable powers of resistance to external influences, and can retain their vitality for a long time outside the body in various conditions; in fact, in this respect they may be said to occupy an intermediate position between spores and spore-free bacilli. Dried phthisical sputum has been found to contain still virulent bacilli after two months, and similar results are obtained when the bacilli are kept in distilled water for several weeks. So also they resist for a long time the action of putrefaction, which is rapidly fatal to many pathogenic organisms. Sputum has been found to contain living tubercle bacilli even after being allowed to putrefy for several weeks (Fraenkel, Baumgarten), and the bacilli have been found to be alive in tubercular organs which have been buried in the ground for a similar period. They are not killed by being exposed to the action of the gastric juice for six hours, or to a temperature of −3° C. for three hours, even when this is repeated several times. It has been found that when completely dried they can resist a temperature of 100° C. for an hour, but, on the other hand, exposure in the moist condition to 70° C. for the same time is usually fatal. It may be stated that raising the temperature to 100° C. kills the bacilli in fluids and in tissues, but in the case of large masses of tissue care must be taken that this temperature is reached throughout. They are killed in less than a minute by exposure to 5 per cent. carbolic acid, and both Koch and Straus found that they are rapidly killed by being exposed to the action of direct sunlight. But it is much more difficult to sterilise sputum containing tubercle bacilli by means of antiseptics, as the physical and chemical characters of the sputum prevent effective penetration of the chemicals used.

Action on the Tissues.—The *local lesion* produced by the tubercle bacillus is the well-known tubercle nodule, the structure of which varies in different situations and according to the intensity of the action of the bacilli. After the bacilli gain

entrance to a connective tissue such as that of the iris, their first action appears to be on the endothelial and connective-tissue cells, which become somewhat swollen and undergo mitotic division, the resulting cells being distinguishable by their large size and pale nuclei—the so-called endothelioid cells. These proliferative changes may be well seen on the fifth day after inoculation or even earlier. A small focus of proliferated cells is thus formed in the neighbourhood of the bacilli, and about the same time numbers of leucocytes—chiefly lymphocytes—begin to appear at the periphery and gradually become more numerous. Soon, however, the action of the bacilli as cell-poisons comes into prominence. The endothelioid cells become swollen and somewhat hyaline, their outlines become indistinct, whilst their nucleus stains faintly, and ultimately loses the power of staining. The cells in the centre, thus altered, gradually become fused into a homogeneous substance, and this afterwards becomes somewhat granular in appearance. If the central necrosis does not take place quickly, then giant-cell formation may occur in the centre of the follicle, this constituting one of the characteristic features of the tubercular lesion ; or after the occurrence of caseation giant-cells may be formed in the cellular tissue around. The centre of a giant-cell often shows signs of degeneration, such as hyaline change and vacuolation, or it may be more granular than the rest of the cell. The exact mode of formation of a tubercle follicle varies, however, in different tissues.

Though there has been a considerable amount of discussion as to the mode of origin of the giant-cells, we think there can be little doubt that in most cases they result from enlargement of single endothelioid cells, the nucleus of which undergoes proliferation without the protoplasm dividing. These endothelioid cells may sometimes be the lining cells of capillaries. Some consider that the giant-cells result from a fusion of the endothelioid cells ; but, though there are occasionally appearances which indicate such a mode of formation, it cannot be regarded as of common occurrence. In some cases of acute tuberculosis, when the bacilli become lodged in a capillary, the endothelial cells of its wall may proliferate, and thus a ring of nuclei may be seen round a small central thrombus. Such an occurrence gives rise to an appearance closely resembling a typical giant-cell. There can be no doubt that the cell necrosis and subsequent caseation depend upon the products of the bacilli, and are not due to the fact that the tubercle nodule is non-vascular. This non-vascularity itself is to be explained by the circumstance that young capillaries cannot grow into a part where tubercle bacilli are active, and that the already existing capillaries become thrombosed, owing to the action of the bacillary products on their walls, and ultimately disappear. At the periphery of tubercular lesions there may be considerable vascularity and new formation of capillaries.

The *general symptoms of tuberculosis*—pyrexia, perspiration, wasting, etc.—are to be ascribed to the absorption and distribution throughout the system of the toxic products of the bacilli ; in the case of phthisical cavities and like conditions where other bacteria are present, the toxins of the latter also play an important part. The occurrence of amyloid change in the organs is believed by some to be chiefly due to the products of other,

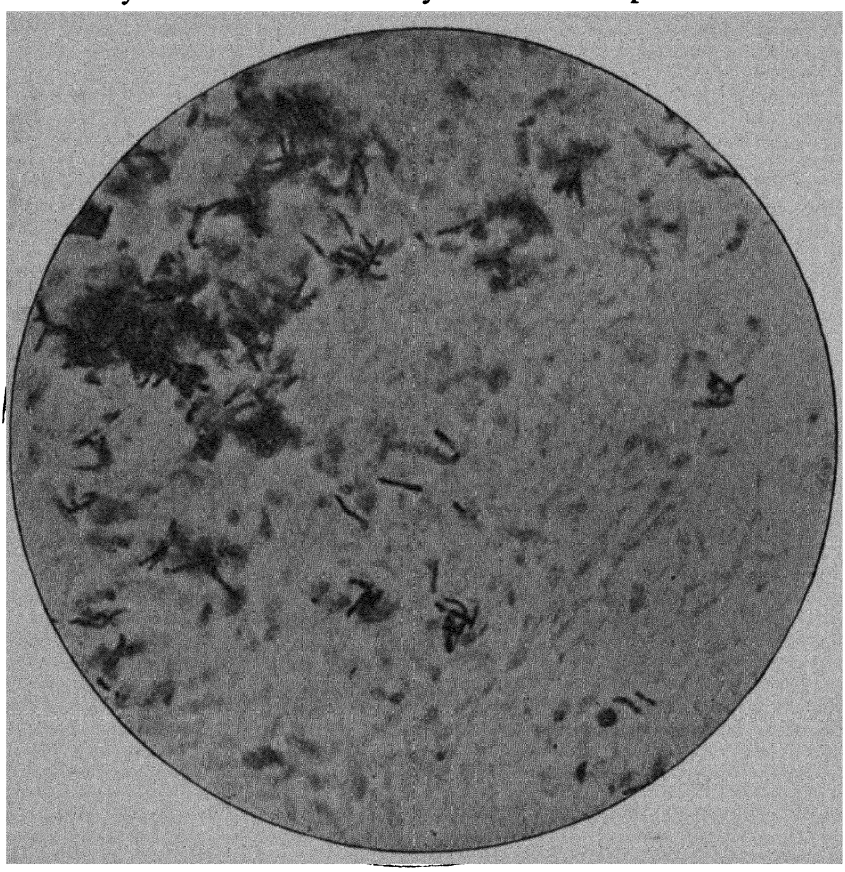


FIG. 76.—Tubercle bacilli in section of human lung in acute phthisis. The bacilli are seen lying singly, and also in large masses to left of field. The pale background is formed by caseous material. Stained with carbol-fuchsin and Bismarck-brown. ×1000.

especially pyogenic, organisms, secondarily present in the tubercular lesions. This matter, however, requires further elucidation.

Presence and Distribution of the Bacilli.—A few facts may be stated regarding the presence of bacilli, and the numbers in which they are likely to be found in tubercular lesions. They are usually very few in number in chronic lesions, whether these are tubercle nodules with much connective-tissue formation or old caseous collections. In caseous material one can

sometimes see a few bacilli faintly stained, along with very minute unequally stained granular points ; but not infrequently none can be detected. The important fact, however, has been established, that tubercular material in which no bacilli can be found microscopically may be proved, on experimental inoculation into animals, to be still virulent. In subacute lesions, with well-formed tubercle follicles and little caseation, the bacilli are

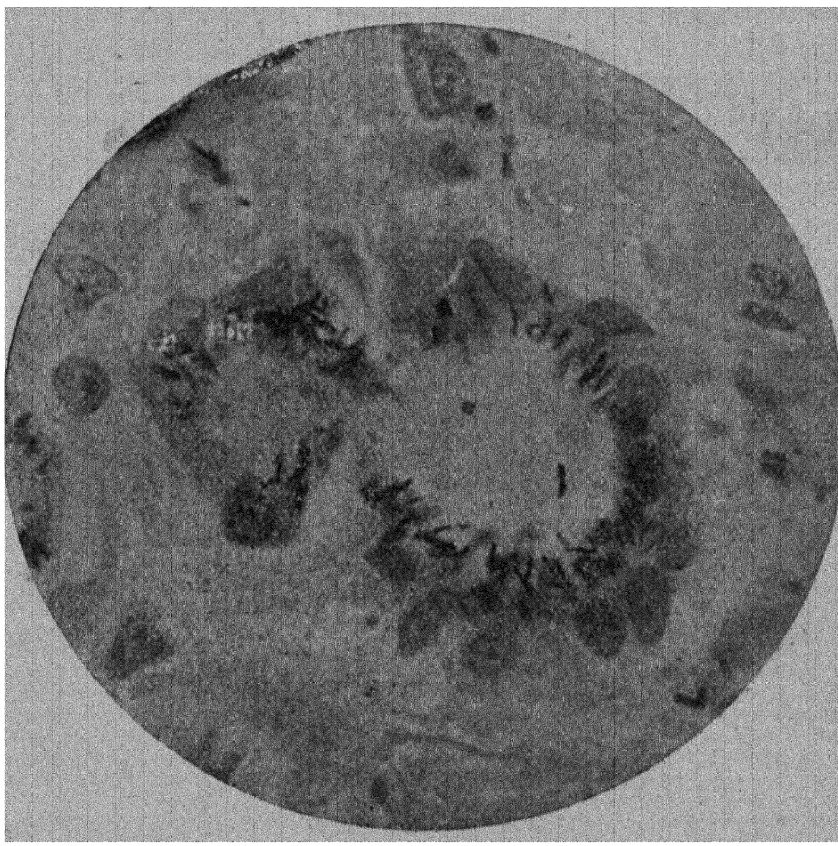


FIG. 77.—Tubercle bacilli in giant-cells, showing the radiate arrangement at the periphery of the cells. Section of tubercular udder of cow.
Stained with carbol-fuchsin and Bismarck-brown. ×1000.

generally scanty. They are most numerous in acute lesions, especially where caseation is rapidly spreading, for example, in such conditions as caseous catarrhal pneumonia (Fig. 76), acute tuberculosis of the spleen in children, which is often attended with a good deal of rapid caseous change, etc. ; in such conditions they often form large masses which are easily seen under a low power of the microscope. In acute miliary tuberculosis a few bacilli can generally be found in the centre of the follicles ; but here they are often much more scanty than one would expect.

The tubercle bacillus is one which not only has comparatively slow growth, but retains its form and staining power for a much longer period than most organisms. As a rule, the bacilli are extracellular in position. Occasionally they occur within the giant-cells, also in endothelioid cells and in leucocytes; the intracellular site is, however, commoner in some of the lower animals. In the ox, for example, tubercle bacilli are commonly found within giant-cells, in which they are often arranged in a somewhat radiate manner at the periphery; it is also common to find bacilli in considerable numbers scattered irregularly throughout the cellular connective tissue of the lesions, even when there is little or no caseation present (Fig. 77).

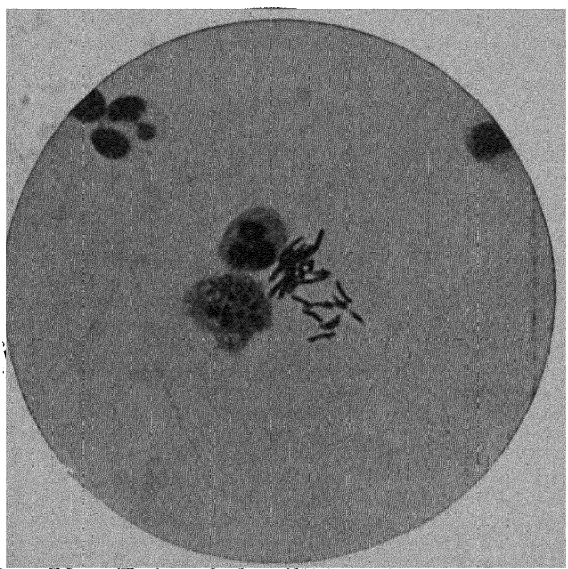


FIG. 78.—Tubercle bacilli in urine; showing one of the characteristic clumps in which they often occur.
Stained with carbol-fuchsin and methylene-blue. ×1000.

In tuberculosis in the horse and in avian tuberculosis the numbers of bacilli may be enormous, even in lesions which are not specially acute; and considerable variation both in their number and in their site is met with in tuberculosis of other animals.

In discharges from tubercular lesions which are breaking down, tubercle bacilli are usually to be found. In the sputum of phthisical patients their presence can be demonstrated almost invariably at some period, and sometimes their numbers are very large (for method of staining, see p. 342). Several examinations may, however, require to be made, also animal inoculation, before any conclusion as to the non-tubercular nature of a case can be drawn. In tubercular meningitis the bacilli can often be found in the cerebro-spinal fluid obtained by lumbar puncture, but are frequently scanty. In cases of genito-urinary tuberculosis they are usually present in the urine; but as they are much diluted it is difficult to find them unless a deposit is obtained by means of the centrifuge. This deposit is examined in the same way as the sputum. The bacilli often occur in little clumps, as shown in Fig. 78. In tubercular ulceration of the

intestine their presence in the fæces may be demonstrated, as was first shown by Koch; but in this case their discovery is usually of little importance, as the intestinal lesions, as a rule, occur only in advanced stages when diagnosis is no longer a matter of doubt.

Experimental Inoculation.—Tuberculosis can be artificially produced in animals in a great many different ways—by injection of the bacilli into the subcutaneous tissue, into the peritoneum, into the anterior chamber of the eye, into the veins; by feeding the animals with the bacilli; and, lastly, by making them inhale the bacilli suspended in the air.

The exact result, of course, varies in different animals and according to the method of inoculation, but we may state generally that when introduced into the tissues of a susceptible animal, the bacilli produce locally the lesions above described, terminating in caseation; that there occurs a tubercular affection of the neighbouring lymphatic glands, and that lastly there may be a rapid extension of the bacilli to other organs by the blood stream and the production of general tuberculosis. Of the animals generally used for the purpose, the guinea-pig is most susceptible.

When a guinea-pig is inoculated subcutaneously with tubercle bacilli from a culture, or with material containing them, such as phthisical sputum, a local swelling gradually forms which is usually well marked about the tenth day. This swelling becomes softened and caseous, and may break down, leading to the formation of an irregular ulcerated area with caseous lining. The lymphatic glands in relation to the parts can generally be found to be enlarged and of somewhat firm consistence, about the end of the second or third week. Later, in them also caseous change occurs, and a similar condition may spread to other groups of glands in turn, passing also to those on the other side of the body. During the occurrence of these changes, the animal loses weight, gradually becomes cachectic, and ultimately dies, sometimes within six weeks, sometimes not for two or three months. *Post mortem*, in addition to the local and glandular changes, an acute tuberculosis is usually present, the spleen being specially affected. This organ is swollen, and is studded throughout by numerous tubercle nodules, which may be minute and grey, or larger and of a yellowish tint. If death has been long delayed, calcification may have occurred in some of the nodules. Tubercle nodules, though rather less numerous, are also present in the liver and in the lungs, the nodules in the latter organs being usually of smaller size though occasionally in

large numbers. The extent of the general infection varies; sometimes the chronic glandular changes constitute the outstanding feature. Statements as to differences in the pathogenic effects of bacilli from human and bovine sources will be found below (p. 325).

Varieties of Tuberculosis.—1. *Human and Bovine Tuberculosis.*—Up till recent years it was generally accepted that all mammalian tuberculosis was due to the same organism, and, in particular, that tuberculosis could be transmitted from the ox to the human subject. The matter became one of special interest owing to Koch's address at the Tuberculosis Congress in 1901, in which he stated his conclusion that human and bovine tuberculosis are practically distinct, and that if a susceptibility of the human subject to the latter really exists, infection is of very rare occurrence—so rare that it is not necessary to take any measures against it. Previously to this, Theobald Smith had pointed out differences between human and bovin tubercle bacilli, th most striking being that the latter possess a much highe virulence to the guinea-pig, rabbit, and other animals, and i particular that human tubercle bacilli, on inoculation int oxen, produce either no disease or only local lesions withou any dissemination. Koch's conclusions were based chiefly o the result of his inoculations of the bovine species with huma tubercle bacilli, the result being confirmatory of Smith's, an also on the supposition that infection of the human subjec through the intestine is of very rare occurrence.

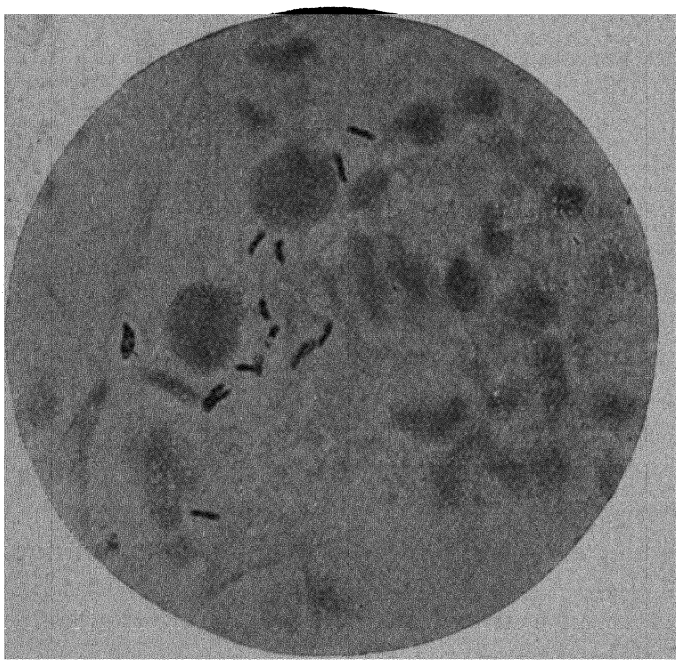


FIG. 79.—Bovine tubercle bacilli in milk. ×1000.

After Koch's communication an enormous amount of wor was done on this subject, and commissions of inquiry wer appointed in various countries. We may summarise the chi

facts which have been established. Practically all observers are agreed that there are two chief types of tubercle bacilli, which differ both in their cultural characters and in their virulence—a *bovine type* and a *human type.* The bacillus of the bovine type (*Mycobacterium tuberculosis bovis*), when cultivated, is usually shorter and thicker and more regular in size (Fig. 79); whilst its growth on various culture media is scantier than that of the human type. From the latter character the British Royal Commission applied the term *dysgonic* to the bovine and *eugonic* to the human type. For distinguishing the growth characters of the two types, egg media (p. 56) are especially suitable. On Dorset's medium the human type produces an abundant, dry, and wrinkled or verrucose growth, which has often a yellowish or pinkish tint; while the bovine type forms a thin whitish layer, smooth or somewhat granular, rather moist in appearance, and the growth is much more easily broken up. The difference between the two types is accentuated by the addition of glycerin to the medium; this greatly favours the growth of the human type, while it does not favour, or even inhibits, the growth of the bovine type. In fact, on glycerin egg medium primary cultures of the latter often fail. These differences are most marked in the early cultures; in later subcultures they tend to diminish. The vitality of the bovine type is less on artificial media, cultures having sometimes a tendency to die out. As already stated, there is also a great difference in virulence towards the lower animals, the bacillus from the ox having a much higher virulence. This organism when injected in suitable quantities into the ox produces a local tubercular lesion, which is usually followed by a generalised and

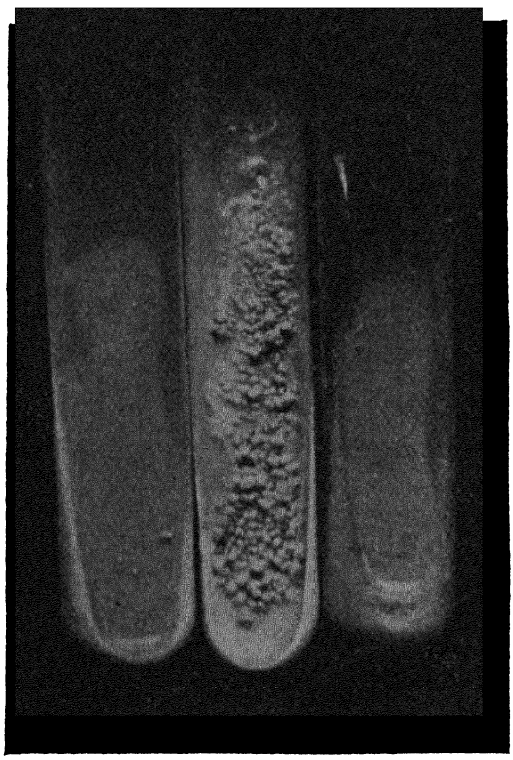


FIG. 80.—Cultures of bovine and human bacilli five weeks old on glycerin egg. The central tube is human, the tube on each side bovine. The three tubes were inoculated on the same day.

fatal tuberculosis; whereas injection of human tubercle bacilli produces no more than a local lesion, which undergoes retrogression. (In certain experiments, *e.g.* those of Delépine, Hamilton, and Young, general tuberculosis has been produced in the bovine species by tubercle bacilli from the human subject, but these results are exceptional.) Corresponding differences come out in the case of the rabbit; in fact, intravenous injection of suitable quantities (*e.g.* of 0·01–0·1 mgrm. of dried bacilli suspended in 1 c.c. of saline) in this animal is the readiest method of distinguishing the two types—an acute tuberculosis resulting with the bovine, but not with the human type. In guinea-pigs and monkeys a generalised tuberculosis may result from subcutaneous injection of bacilli of the human type, but in this case also the difference in favour of the greater virulence of the bovine type is made out. With regard to the distribution of the two types of organisms, it may be stated that, so far as we know, the bacillus obtained from bovine tuberculosis is always of the bovine type; in fact, this seems to be the prevalent organism in animal tuberculosis (*vide infra*). In human tuberculosis the bacilli in a large majority of the cases are of the human type; but, on the other hand, in a certain proportion bacilli of the bovine type are present. Pulmonary phthisis is almost invariably caused by bacilli of the human type; a few cases have been recorded in which the bovine type has been present, but these constitute less than 1 per cent. of the cases investigated. The Royal Commission found that the bovine type was present in 50 per cent. of cases of primary abdominal tuberculosis in children—that is, in cases where apparently infection had taken place by alimentation; more recent observations have shown that the proportion of cases of glandular tuberculosis in children under ten years of age produced by bovine bacilli varies considerably in different localities. In cases of lupus nearly half of the bacilli obtained were of the bovine type, and it is an interesting fact that almost all the viruses, both of the human and bovine types, were markedly attenuated in their virulence for animals. In over two hundred cases of tuberculosis in children, given by W. H. Park, the bovine bacillus was present in more than 25 per cent., the percentage being higher in the earlier than in the later years of childhood; and Fraser found that of seventy cases of tuberculosis of bones and joints in children in Edinburgh, this was the type present in more than half. This proportion is higher than that found by Eastwood and F. Griffith and by A. Stanley Griffith in a large number of cases, chiefly in England, namely, a little over 25 per

cent. Fraser also found that the proportion of cases in which the bovine type is present is much higher when there is no evidence of infection from other members of the family, than when there is the possibility of such infection. Almost all the tubercular lesions from which the bovine type has been obtained have been in children, the presence of the bovine type of bacillus in adult tubercular lesions, phthisical sputum, etc., being of very rare occurrence. It is therefore justifiable to conclude that tuberculosis is transmissible from the ox to man, and that the milk of tubercular cows is a common vehicle of transmission.

Although most of the bacilli which have been cultivated correspond to one of the two types, as above described, it is also to be noted that intermediate varieties are occasionally met with, though some of these on analysis have been found to be really due to a mixture of the two types. According to some observers, it is possible to modify bacilli of the human type by passing them through the bodies of certain animals, *e.g.* guinea-pigs, sheep, and goats, so that they acquire the characters of bovine bacilli, but the more recent results, confirming those of the Royal Commission, are that this modification does not take place and that the characters of the type are comparatively stable. The question is still an open one, and it is doubtful whether or not a bovine type after long sojourn in the human tissues will assume the characters of the human type ; if it does, the proportion of cases actually due to the bovine type will be of course larger than is indicated by the characters of the organism obtained from the lesion. It is quite likely that, although the bovine bacilli are more virulent to the lower animals than the human bacilli are, this does not hold also in the case of the human subject. In fact, the comparative chronicity of the primary abdominal lesions in children, in the first instance, would point rather to a low order of virulence towards the human subject. We may also add that there are cases, notably those of Ravenel, in which accidental inoculation of the skin in the human subject with bovine tubercle has resulted in the production of tuberculosis.

Some other facts obtained by the Royal Commission may be given. The bovine type of bacillus alone was found in the sheep, goat, and horse, whilst in the pig the bovine type was found in the great majority of cases, though in some the human type, and in others the avian tubercle bacillus, was present. In the case of these two latter the lesions were of a more localised kind. The bovine type was also found in the cat. The human type was found in animals in confinement, *e.g.* the antelope, gnu, chimpanzee, and Macacus rhœsus, and also in the parrot. The animals most susceptible to inoculation with the human type are the guinea-pig, rhœsus, and chimpanzee ; the dog, rat, and mouse are relatively resistant, while the calf, rabbit, pig, and goat occupy an intermediate position. The parrot also has been found to be susceptible to inoculation with the human type. It was also shown that when cows were inoculated subcutaneously with considerable quantities of bacilli either of the human or bovine type the bacilli were excreted in the milk, and that in these cases the udder appeared normal. There is therefore the

presumption that when during the course of the disease the bacilli are present in the blood stream, they may make the milk infective even though there are no discoverable lesions in the udder.

2. *Avian Tuberculosis.*—In the tubercular lesions in birds there are found bacilli (*Mycobacterium avium*) which correspond in their staining reactions and in their morphological characters with those in mammals, but differences are observed in cultures, and also on experimental inoculation.

On glycerin agar and on serum, the growth of tubercle bacilli from birds is more luxuriant, has a moister appearance (Fig. 75, C), and, moreover, takes place at a higher temperature, 43·5° C., than is the case with mammalian tubercle bacilli. Experimental inoculation brings out even more distinct differences Tubercle bacilli derived from the human subject or from the ox, for example, when injected into fowls, usually fail to produce tuberculosis, whilst those of avian origin very readily do so (on the other hand, the parrot is susceptible to inoculation with both mammalian types). Fowls are also very susceptible to the disease when fed with portions of the organs containing avian tubercle bacilli, but they can consume enormous quantities of phthisical sputum without becoming tubercular (Straus, Wurtz, Nocard). The Royal Commission found that rabbits and mice are the only mammals susceptible to inoculation with avian tubercle bacilli, though others may succumb to toxic effects when large doses are used. In the case of the rabbit, intravenous injection results in the formation of greyish-white foci in the spleen, but no true tubercles are formed ; subcutaneous inoculation leads to a peculiar chronic disease in joints, testes, etc., whilst the liver and spleen are free from lesions—a result not obtained with mammalian bacilli.

There is, therefore, abundant evidence that the bacilli derived from the two classes of animals show important differences, and, reasoning from analogy, we might infer that probably the human subject also would be little susceptible to infection from avian tuberculosis. The question remains—Are these differences of a permanent character ? Nocard found that mammalian bacilli of the human type, when kept within closed collodion sacs in the peritoneal cavities of fowls over a long period of time, acquired the characters of avian bacilli, but the Royal Commission, as the result of similar experiments, obtained no evidence of such transformation. It is accordingly not possible at present to give a definite answer to the question.

3 *Tuberculosis in the Fish.*—Bataillon, Dubard, and Terre cultivated from a tubercle-like disease in a carp, a bacillus (*Mycobacterium piscium*) which, in staining reaction and microscopic characters, closely agrees with the tubercle bacillus. The lesion with which it was associated was an abundant growth of granula-

tion tissue in which numerous giant-cells were present. It forms, however, luxuriant growth at room temperature, the growth being thick and moist like that of avian tubercle bacilli (Fig. 82, *c*). Growth does not occur at the body temperature, though by gradual acclimatisation a small amount of growth has been obtained up to 36° C. Furthermore, the organism appears to undergo no multiplication when injected into the tissues of mammals, and attempts to modify this characteristic have so far been unsuccessful. Weber and Taute have cultivated a similar organism from mud, and also from organs of healthy frogs. It is thus probably to be regarded as a saprophyte which is only occasionally associated with disease in the fish.

According to the results of different experimenters, it is possible to modify human tubercle bacilli by allowing them to sojourn in the tissues of cold-blooded animals, *e.g.* the frog, blind-worm, etc., so that they flourish at lower temperatures. These results have, however, been called in question, as it has been stated the organisms obtained were not modified tubercle bacilli, but other acid-fast bacilli which may be found in the tissues of normal cold-blooded animals. This question must accordingly be considered still an open one.

Other Acid-fast Bacilli.—Within recent years a number of bacilli presenting the same staining reaction as the tubercle bacilli have been discovered. Such bacilli have a comparatively wide distribution in nature, as they have been obtained from various species of grass, from butter and milk, from manure, and from the surfaces of animal bodies. Microscopically, they agree more or less closely with tubercle bacilli, though most of them are shorter and plumper; many of them show filamentous and branching forms under certain conditions of culture. Moreover, on injection, they produce granulation tissue nodules which may resemble tubercles, although on the whole there is a greater tendency to softening and suppuration, and usually the lesions are localised to the

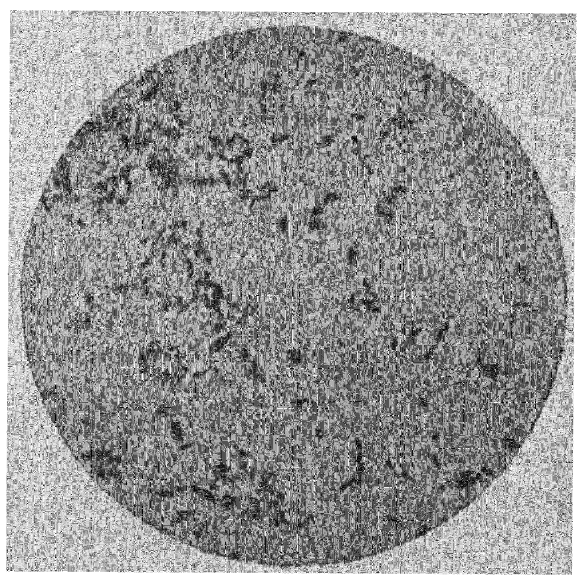
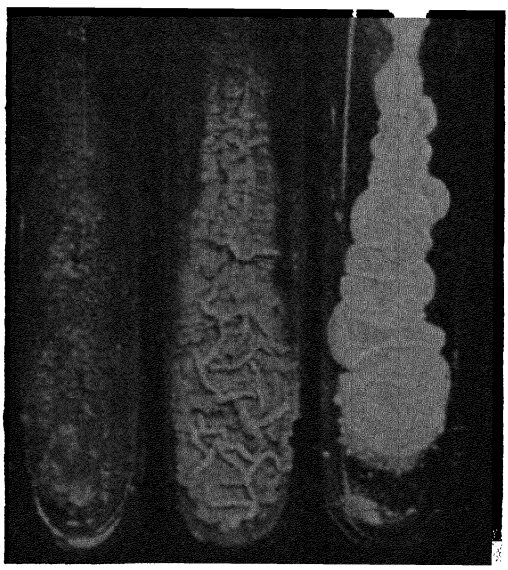


FIG. 81.—Moeller's Timothy-grass bacillus. From a culture on agar. Stained with carbol-fuchsin, and treated with 20 per cent. sulphuric acid. ×1000.

site of inoculation. The most important point of distinction is the fact that their multiplication on artificial media is much more rapid, growth usually being visible within forty-eight hours and often within twenty-four hours at 37° C. Furthermore, in most instances growth occurs at the room temperature and ordinary agar is a suitable medium. The general character of the cultures in this group is a somewhat irregular layer, often with wrinkled surface, dry or moist in appearance, and varying in tint from white to yellow or reddish-brown.



a *b* *c*

FIG. 82.—Cultures of acid-fast bacilli grown at room temperature.

(*a*) Moeller's Timothy-grass bacillus I.
(*b*) The Petri-Rabinowitsch butter bacillus.
(*c*) Bacillus of fish tuberculosis.

As examples of acid-fast saprophytes we may mention Moeller's grass bacillus (*Mycobacterium phlœi*) (Fig. 82, *a*) (isolated from infusions of Timothy grass), Petri's (*M. butyricum*), and Rabinowitsch's (*M. berolinense*) butter bacilli (Fig. 82, *b*), the "Mist (dung) bacillus" of Moeller (*M. stercoris*), etc. Kolle and his co-workers have stated that as a result of repeated animal passage certain of the saprophytic acid-fast organisms became enhanced in virulence until they behaved like tubercle bacilli; at the same time their cultural characters also became altered so that finally they resembled the latter. It may be said, however, that at present it is generally held that the tubercle bacilli and the acid-fast saprophytic organisms are distinct species, and that therefore the latter are not a source of danger in the spread of tuberculosis.

Johne's Bacillus.—Another bacillus of considerable interest is Johne's bacillus (*Mycobacterium paratuberculosis*) or the bacillus of "chronic bovine pseudo-tuberculous enteritis," the lesions produced by it being corrugated thickenings of the mucous membrane, especially of the small intestine. The disease has now been observed in various countries, and has been found to be comparatively common in Britain. The bacilli occur in large numbers in the lesions, the cells being often packed with them, and can readily be found in scrapings from the surface. They resemble the tubercle bacillus in appearance, but are distinctly shorter; they are equally acid-fast. The organism has been cultivated by Twort and Ingram on egg medium to which there is added ½–1 per cent. of dried and powdered acid-fast bacilli, the Timothy-grass bacillus being most

suitable; growth is slow, the colonies appearing after about four weeks in the primary cultures.

Smegma Bacillus (*Mycobacterium smegmatis*).—This organism is of importance, as in form and staining reaction it somewhat resembles the tubercle bacillus and may be mistaken for it. It occurs often in large numbers in the smegma præputiale and in the region of the external genitals, especially where there is an accumulation of fatty matter from the secretions. Morphologically it is a slender, slightly curved organism, like the tubercle bacillus, but usually distinctly shorter (Fig. 83). Like the tubercle bacillus, it stains with some difficulty and resists decolorisation with strong mineral acids. Most observers ascribe the latter fact to the fatty matter with which it is surrounded, and find that if the specimen is treated with alcohol the organism is easily decolorised. Czaplewski, however, who has cultivated it on various media, finds that in culture it shows resistance to decolorisation both with alcohol and with acids, and considers, therefore, that the reaction is not due to the surrounding fatty medium. We have found that in smegma it can be readily decolorised, as a rule, by a minute's exposure to alcohol after the usual treatment with sulphuric acid, and thus it can be readily distinguished from the tubercle bacillus. We, moreover, believe that minor points of difference in the microscopic appearances of the two organisms are quite sufficient to make the experienced observer suspicious if he should meet with the smegma bacillus in urine, and lead him to apply the decolorising test. Difficulty will only occur when a few scattered bacilli retaining the fuchsin are found, or if they are seen to be enclosed in a matrix of hyaline material. The preparation of stained films after treatment of the urinary sediment with antiformin is of value in such cases, as organisms which lose their acid-fast properties in the process are not true tubercle bacilli.

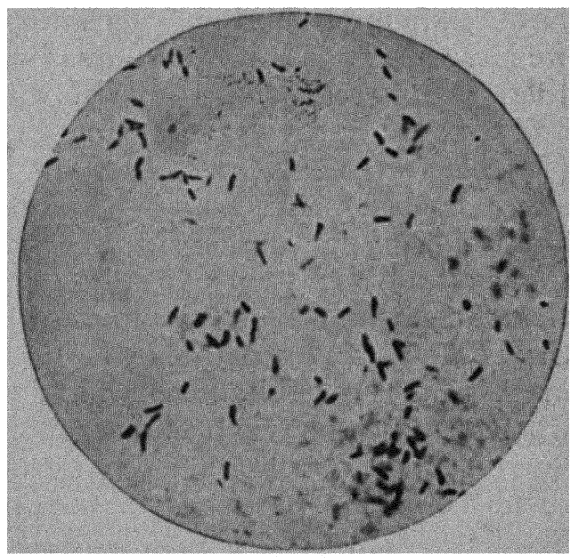


FIG. 83.—Smegma bacilli. Film preparation of smegma. Ziehl-Neelsen stain. ×1000.

Its cultivation, which is attended with some difficulty, was first effected by Czaplewski. On serum it grows in the form of yellowish-grey, irregularly rounded colonies about 1 mm. in diameter, sometimes becoming confluent to form a comparatively thick layer. He found that it also grew on glycerin agar and in bouillon. It is non-pathogenic to various animals which have been tested, unless very large doses are used.

Cowie has found that acid-fast bacilli are of common occurrence in the secretions of the external genitals, mammæ, etc., in certain of the lower animals, and that these organisms vary in appearance.

He considers that the term "smegma bacillus" probably represents a number of allied species.

The question may be asked—Do these results modify the validity of the staining reaction of tubercle bacilli as a means of diagnosis? The source of any acid-fast bacilli in question is manifestly of importance, and it may be stated that when these have been obtained from some source outside the body, or where contamination from without has been possible, their recognition as tubercle bacilli cannot be established by microscopic examination alone. In the case of material coming from the interior of the body, however,—sputum, etc.,—the condition must be looked on as different, and although an acid-fast bacillus (not tubercle) has been found by Rabinowitsch in a case of pulmonary gangrene, we have not sufficient data for saying that acid-fast bacilli other than the tubercle bacillus flourish within the tissues of the *human* body, except in such rare instances as to be practically negligible. (To this statement the case of the leprosy bacillus is of course an exception.) Accordingly, up till now, the microscopic examination of sputum, etc., cannot be said to have its validity shaken, and we have the results of enormous clinical experience that such examination is of practically unvarying value. Nevertheless, the facts established with regard to other acid-fast bacilli must be kept carefully in view, and great care must be exercised when only one or two bacilli are found, especially if they deviate in their morphological characters from the tubercle bacillus. In such cases inoculation may be the only reliable test.

Action of Dead Tubercle Bacilli.—The remarkable fact has been established by independent investigators, that tubercle bacilli in the dead condition, when introduced into the tissues in sufficient numbers, can produce tubercle-like nodules. Prudden and Hodenpyl, by intravenous injection in rabbits of cultures sterilised by heat, produced in the lungs small nodules in which giant-cells, but no caseation, were occasionally present, and which were characterised by more growth of fibrous tissue than in ordinary tubercle. The subject was very fully investigated, with confirmatory results, by Straus and Gamaleia, who found that, if the numbers of bacilli introduced into the circulation were large, there resulted very numerous tubercle nodules with well-formed giant-cells, and occasionally traces of caseation. The bacilli can be well recognised in the nodules by the ordinary staining method. Similar nodules can be produced by intraperitoneal injection. Subcutaneous injection, on the other hand, produces a local abscess, but in this case no secondary tubercles are found in the internal organs. Further, in many of the animals inoculated by the various methods, a condition of marasmus sets in and gradually leads to a fatal result. These experiments, which have been confirmed by other observers, show

that even after the bacilli are dead they preserve their staining reactions in the tissues for a long time, and also that there are apparently contained in the bodies of the dead bacilli certain substances which act locally, producing proliferative and, to a less extent, degenerative changes, and which also markedly affect the general nutrition. S. Stockman has found that an animal inoculated with large numbers of dead tubercle bacilli afterwards gives the tuberculin reaction.

Practical Conclusions.—From the facts above stated with regard to the conditions of growth of the tubercle bacilli, their powers of resistance, and the paths by which they can enter the body and produce disease (as shown by experiment), the manner by which tuberculosis is naturally transmitted can be readily understood. Though the experiments of Sander show that tubercle bacilli can multiply on vegetable media to a certain extent at warm summer temperature, it is doubtful whether all the conditions necessary for growth are provided to any extent in nature. At any rate, the great multiplying ground of tubercle bacilli is the animal body; and tubercular tissues and secretions containing the bacilli are the chief, if not the only, means by which the disease is spread. The tubercle bacilli leave the body in large numbers in the sputum of phthisical patients, and when the sputum becomes dried and pulverised they are set free in the air. As examples of the extent to which this takes place, it may be said that their presence in the air of rooms containing phthisical patients has been repeatedly demonstrated. Williams placed glass plates covered with glycerin in the ventilating shaft of the Brompton Hospital, and after five days found, by microscopic examination, tubercle bacilli on the surface, whilst Klein found that guinea-pigs kept in the ventilating shaft became tubercular. Cornet produced tuberculosis in rabbits by inoculating them with dust collected from the walls of a consumptive ward. Tubercle bacilli are also discharged in considerable quantities in the urine in tubercular disease of the urinary tract, and also by the bowel when there is tubercular ulceration; but, so far as the human subject is concerned, the great means of disseminating the bacilli in the outer world is dried phthisical sputum, and the source of danger from this means can scarcely be overestimated.

Another great source of infection is the milk of cows affected with tuberculosis of the udder, and this is responsible for a considerable proportion of tuberculosis of lymphatic glands, bones, and joints, etc., in young children, as above detailed. In the examination of milk, animal inoculation with centrifuged samples is the only reliable means of detecting the

presence of tubercle bacilli. As pointed out by Woodhead and others, the milk from cows thus affected is probably the great source of tabes mesenterica, which is so common in young subjects (*vide* p. 326). In these cases there may be tubercular ulceration of the intestine, or it may be absent. It is especially in children that this mode of infection occurs, as in the adult ulceration of the intestine is rare as a primary infection, though it is common in phthisical patients as the result of infection by the bacilli in the sputum which has been swallowed. There is less risk of infection by means of the flesh of tubercular animals, for, in the first place, tuberculosis of the muscles of oxen being very rare, there is little chance of the bacilli being present in the flesh unless the surface has been smeared with the juice of the tubercular organs, as in the process of cutting up the parts; and, in the second place, even when present they will be destroyed if the meat is thoroughly cooked.

We may state, therefore, that the two great modes of infection are by inhalation, and by ingestion, of tubercle bacilli. In the former, the tubercle bacilli will in most cases be derived from the human subject; in the latter, probably from tubercular cows, though inhaled tubercle bacilli may also be swallowed and contamination of food by tubercular material from the human subject may occur. Alike when inhaled and when ingested, tubercle bacilli may lodge about the pharynx and thus come to infect the pharyngeal lymphoid tissue, tonsils, etc., tubercular lesions of these parts being much more frequent than was formerly supposed. Thence the cervical lymphatic glands may become infected, and afterwards other groups of glands, bones, or joints, and internal organs. It is to be noted that there is a predilection for the lungs by whatever route the bacilli enter the body, and accordingly that pulmonary lesions are not always the result of inhalation.

The Specific Reactions of Tubercle Bacilli.—The account of the effects of the tubercle bacillus given above shows that it produces toxic substances which both act on the tissues around and also affect the body generally. Nevertheless the organism does not to any extent produce separable soluble toxins in fluid media; the toxic substances are bound up with the substance of the bacteria, that is, are endotoxins. Tuberculin as first introduced by Koch in 1890 consisted of the disintegration products of the bacilli and thus contained endotoxins along with other substances. He showed that in tuberculous animals there was supersensitiveness to tuberculin, which was shown by a *local* reaction at the site of introduction of the tuberculin, by a *general*

reaction, indicated by malaise, pyrexia, etc., and also by a *focal* inflammatory reaction around the tuberculous lesions. These reactions accordingly supply a means by which the presence of tuberculous foci in the body can be recognised. Koch supposed that the focal reaction might lead to encapsulation of the tuberculous lesion and thus to cure, or, if the lesion were superficial, to its being ulcerated and cast off. Tuberculin could thus be applied for therapeutic as well as diagnostic purposes. It soon came to be recognised, however, that the administration of tuberculin in the doses then used (doses sufficient to produce a general reaction) might be attended by harm, and the use of tuberculin in this way came to be abandoned. At a later period treatment by tuberculin was resumed but was carried out with very much smaller doses, in fact with doses insufficient to produce systemic disturbance; and favourable results have been claimed. Tuberculin employed in this way may act as a stimulant of the tissue around the tubercular lesions and thus be beneficial; it may aid also by leading to the production of specific antibodies. But in any case it must be regarded as only an auxiliary agent, and even then only when employed with care and over a considerable period. (For varieties of tuberculin, *vide* p. 341.)

When the presence of antibodies came to be recognised in infections and in the state of immunity, search for specific antibodies was carried out and various forms of these were found in tuberculous infections. Certain facts have been established with regard to them, but it must be admitted that they are not such as readily supply much assistance in diagnosis. We shall now give an account in greater detail under the two headings of supersensitiveness and immunity, though these two are probably related in essence.

Phenomena of Supersensitiveness.—TUBERCULIN IN DIAGNOSIS.—The original method employed by Koch was that of subcutaneous injection of tuberculin, and with it all types of reaction—local, general, and focal—are seen. At a later period methods involving the use of much smaller amounts of tuberculin were introduced; the criterion in these is simply local reaction due to tissue allergy, the doses employed being insufficient to bring about the other types of reaction. These methods may now be briefly described.

Subcutaneous method (Koch).—A positive reaction may be produced in tubercular subjects by the injection of 0·001–0·0001 c.c. of the original tuberculin. A local inflammatory swelling occurs at the site of injection, and this is accompanied by a rise of temperature, usually of a short character. There is also a focal

reaction around any tubercular lesion, and if the lesion is visible, *e.g.* lupus, this is indicated by an inflammatory redness, which may be followed by ulceration. In the case of a pulmonary lesion there may be signs of irritation—tendency to cough, increased expectoration, increase of râles in the chest, etc.

Cutaneous Test of von Pirquet.—This is carried out as follows : The skin, usually that of the flexor aspect of the forearm, is well cleansed with ether and then allowed to dry. Two drops of tuberculin are placed on the prepared surface about 4 inches apart, and then midway between the two drops a small spot is scarified with a small metal borer constructed for the purpose ; in the process only the epidermis should be injured and blood should not be drawn. This serves as a control, any reaction which follows in it being merely a traumatic one. Similar scarification is effected through the drops of tuberculin, so that the scarified spots are exposed to its action. Small portions of cotton wool are placed over the drops to prevent the tuberculin from running off, and the latter is allowed to act for ten minutes. After that time the cotton wool is removed ; no dressing is required. The " old " tuberculin of Koch is that used. In the case of a positive reaction an inflammatory redness and swelling make their appearance round the sites of tuberculin inoculation, generally within a few hours, and at the end of twenty-four hours there is a distinct inflammatory papule about half an inch in diameter, with a somewhat paler centre like a spot of urticaria ; sometimes in the centre there are minute vesicles. The maximum effect usually occurs within forty-eight hours, and after that time the reaction gradually recedes. Such is the typical reaction, but of course slighter, and also more intense reactions are met with. In a negative reaction all three points of scarification show merely a slight traumatic redness which soon passes off.

Intracutaneous Method of Mantoux.—In this modification 0·1 c.c. of diluted tuberculin is injected into the cutis by means of a fine needle. The doses ordinarily used are 0·0001–0·00001 gram of tuberculin, that is 0·1 c.c. of dilutions 1 : 1000–1 : 10,000. At the site of injection, a positive reaction is shown by the occurrence of redness, inflammatory œdema, and sometimes the formation of a vesicle. The reaction occurs within a few hours and reaches the maximum on the following day ; thereafter it gradually passes off.

The ophthalmo-reaction of Calmette.—Koch's original tuberculin is precipitated with 95 per cent. alcohol ; the precipitate is then dissolved in water. This process is repeated other two times, and

the final precipitate is made up as a 1 per cent. solution in distilled water. For use in the case of an adult, a drop of this solution is placed in the conjunctival sac and the fluid allowed to spread over the surface ; for children, about half this quantity is sufficient. In the case of a positive reaction the ocular conjunctiva is congested, the lids become somewhat swollen and their inner surface presents a bright red colour, there is increased secretion of tears and a varying amount of exudation. The reaction usually reaches its maximum in from six to ten hours after the instillation, and commences to pass off in from twenty-four to thirty-six hours—in children a little sooner.

The general results obtained by these reactions appear to correspond closely. A distinct positive result obtained by either is nearly conclusive as to the presence of a tubercular lesion, though not necessarily of active nature. In cases of latent tuberculosis the reaction is sometimes obtained, sometimes not. Again, in very advanced cases of tuberculosis, especially a short time before death, a negative result may be got ; in some of these cases v. Pirquet has met with a colourless papule or a livid spot without exudation, conditions which he describes as indicating a " cachectic reaction." The cutaneous and intracutaneous methods are those which are now most frequently used. The former is the more readily applied, but the latter is the more delicate. The ophthalmo reaction also is easily applied, at least in adults, but its use is contra-indicated when there is any abnormal condition of the conjunctiva. Even apart from this, however, inflammatory symptoms of disagreeable severity sometimes supervene, and the test is now not much used. It should also be noted that a second test ought not to be applied to the same eye, as the first may produce a condition of supersensitiveness (p. 229).

The Use of Old Tuberculin in the Diagnosis of Tuberculosis in Cattle. —In cattle, tuberculosis may be present without giving rise to apparent symptoms. It is thus important from the point of view of human infection that an early diagnosis should be made. The method is applied as follows : The animals are kept twenty-four hours in their stalls, and the temperature is taken every three hours, from four hours before the injection till twenty-four after. The average temperature in cattle is 102·2° F. ; 30 to 40 centigrammes of tuberculin are injected subcutaneously, and if the animal be tubercular the temperature rises 2° or 3° F. in eight to twelve hours, and continues elevated for ten to twelve hours. Bang, who has worked most at the subject, lays down the principle that the more nearly the temperature approaches 104° F. the more reason for suspicion is there. He gives a record of 280 cases where the value of the method was tested by subsequent post-mortem examination. He found that with proper precautions the error was only 3·3 per cent.

The method has been largely practised in all parts of the world, and is of great value. It may be noted that if a positive reaction is present it may disappear after repeated injections.

TUBERCULIN THERAPY.—In tuberculosis we meet with certain phenomena unlike those in acute infections against which a solid immunity may be acquired. In the first place, the local reaction around the bacilli plays a very important part, as it represents a struggle between the tissues and the bacilli, in which either may be successful. It is now a well-recognised fact that infection and subsequent cure take place in the early years of life in a large proportion of individuals, and that cure may follow even when the lesion has been of considerable extent. The tissue reaction around the bacilli tends to localise them and also their products, and, at the same time, the endotoxins are bound up in the resistant bodies of the bacteria. The conditions for diffusion of bacterial products are thus unfavourable, and it may be that healing results merely from local reaction without a general immunity being established. Nevertheless, the facts with regard to the tuberculin reaction point to a general effect on the tissues of the body brought about by the bacillary products, and it is generally assumed that healing of tuberculosis in early life brings with it a certain degree of immunity. Evidence of healed tuberculosis, most frequently in the lungs, is very often found in adults, and it is supposed that the appearance of active disease is often due to the lighting up of a dormant lesion. It may also be due, however, to fresh infection, and this is indicated by the fact that in practically all cases of adult tuberculosis the bacilli present are of the human type. In either case, in the development of active disease depressed vitality and like conditions are recognised as important factors, overcoming any immunity and leading to susceptibility. It is possible that the result may vary also according to the virulence of the bacilli, but with regard to this there is little known.

From what has been stated, however, it will appear that there are two possible means of therapeutic action, namely, on the one hand, by aiding in the local defence of the tissue, and on the other, by stimulating the production of antibodies. Unfortunately, even when the latter occurrence is attained we do not know their significance in relation to the establishment of effective immunity. Koch introduced tuberculin-R as a substance which was supposed to have the minimum necrotic effect on tubercular foci, and it was used for the purpose of

increasing the general resisting powers of the tissues. Even at the present time, however, it is not possible to recognise two different elements in a tuberculin, leading to the reactive effects on the one hand and the immunising effects on the other.

The need for a standard to control treatment by tuberculin has long been recognised, and for this purpose Wright introduced the estimation of the opsonic index. The general principle advocated by him was to proceed by such stages as to raise the opsonic index, which is usually low in localised tuberculosis, and for this purpose he formulated certain rules in treatment. The method has now, however, been practically abandoned. The general principle now usually adopted in treatment with tuberculin is to commence with small doses and to increase them gradually at suitable intervals of time, but always to avoid constitutional reaction. In this way a tolerance to tuberculin is developed. Another method is to administer very small doses at suitable intervals without giving rise to tolerance, each injection being supposed to raise the opsonic index for a time. (For details reference must be made to special works.) While undoubtedly in many cases good results have been obtained by the use of tuberculin, every administration must be looked upon as of experimental nature, and the treatment should be only in the hands of those who have had experience of the subject. The fact that in so many cases tuberculous infections tend to disappear under ordinary treatment, makes it at present extremely difficult to estimate truly the therapeutic effects of tuberculin treatment.

Immunity Phenomena in Tuberculosis.—The serum of individuals affected with tuberculosis has been investigated for the presence of specific antibodies; and agglutinins, precipitins, opsonins, and complement-fixing immune-bodies have all been demonstrated. Bordet and Gengou showed originally that guinea-pigs which had received subcutaneous injections of avian tubercle bacilli (to which these animals are relatively insusceptible), developed in their serum complement-fixing antibodies for both avian and human types of bacilli. A similar result followed injections of the human type killed by heating at 70° C. and later of dried bacilli. On the other hand, no antibodies were demonstrable at any stage of the infection in animals inoculated with living, virulent, human bacilli. It has been found also that complement-fixing antibodies (immune-bodies) may occur in the serum of human cases of tuberculosis, and that they occur in largest amount in chronic infections of fairly wide extent. On the other hand, they may be absent when

the lesion is encapsulated or quiescent and also when the disease is advancing rapidly, and especially towards the fatal termination. The results obtained, however, have not been such as to be of much clinical value.

Whilst agglutinins may be demonstrated in the blood of tuberculous patients, it is not possible to correlate the agglutinating power with the degree of immunity present. Agglutinins obtained by injecting animals with dead bacilli have been employed for another purpose, namely, to compare different strains of bacilli. Tulloch and his co-workers found that the strains of the human tubercle bacillus formed one well-defined serological group, there being no evidence of the existence of multiplicity of types. G. S. Wilson came to the same conclusion, and found also that bovine and human strains are indistinguishable by agglutination tests. Reference has already been made to the existence of opsonins and to the possibility of raising the opsonic index in tuberculous patients by means of tuberculin. It may be added that antibodies may also result from the injection of large doses of tuberculin in normal animals. But it is not clear whether, and if so to what extent, the presence of antibodies can be taken as an indication of increased resistance to tuberculosis. In this connection it is of interest to note that Laidlaw and Dudley have found that the gum-like substance extracted by them from tubercle bacilli gives a precipitate with a tubercle antiserum. The serum was obtained from a horse immunised by means of Dreyer's diaplyte antigen. The gum-like substance does not, however, act as an antigen, no precipitin being developed when it is injected into animals. In this respect there is an analogy to what has been found in the case of heterophile antigens (p. 200).

It may be taken as established that to confer any considerable degree of acquired immunity against tuberculosis, it is necessary to inoculate the individual with living, although attenuated, tubercle bacilli; and this resistance probably disappears soon after the body ceases to harbour the living organisms. In accordance with this view, many attempts have been made to attenuate tubercle bacilli in various ways. Calmette and his co-workers have obtained successful results by immunising animals with a culture of bovine tubercle bacilli which has been grown for many years on a medium containing bile. The vaccine may be administered by mouth as well as intravenously. Immunity conferred by the use of such attenuated cultures is of relatively short duration, about eighteen months, in cattle. Recent observations by Wilbert show that Calmette's

method affords protection to monkeys in confinement against natural infection by contact from tuberculous animals. These results go to support the view that the healing of a tubercular lesion in earlier life leads to a certain amount of immunity. They are also in accordance with the fact first brought forward by Koch that a tuberculous animal reacts differently from a normal animal to a fresh infection. In the former the resulting lesions remain localised, while in the latter there may be a spreading infection. In view of all the facts, it seems very doubtful whether the presence of a real active immunity can be inferred from the content of the serum in antibodies.

Varieties of Tuberculin. — (1) *Koch's Old Tuberculin.* — This consists of a six-weeks-old culture of tubercle bacilli in 5 per cent. glycerin bouillon, evaporated down to a tenth of its original volume, killed by heat, and filtered.

(2) *Tuberculin-O.*—Masses of living bacillary growth from surface cultures on agar are dried *in vacuo*, ground in an agate mill, treated with distilled water and centrifuged; the supernatant clear fluid is the tuberculin. As it gave no cloudiness on the addition of glycerin, Koch concluded that it contained the glycerin-soluble products present in the "old tuberculin," which were looked on as responsible for the necrotic effects produced by the latter (*vide supra*).

(3) *Tuberculin-R.*—The deposit in the preparation of tuberculin-O is again ground up in distilled water, centrifuged, and the clear fluid set aside; the process is repeated with the residue until, on centrifuging, none is left. The successive supernatant fluids are mixed and concentrated, and constitute the tuberculin. As this fluid gives a cloudiness with glycerin, Koch considered it contained the glycerin-insoluble constituents of the "old tuberculin."

(4) *Koch's New Tuberculin* (*Bazillenemulsion*).—A bacillary mass is dried and ground in 50 per cent. glycerin in water till a clear fluid results. This tuberculin is thus equivalent to a mixture of tuberculin-O and tuberculin-R.

(5) *Tuberculin Bêraneck.*—This preparation is an extract of tubercle bacilli with 1 per cent. phosphoric acid, the effect of which is supposed to be to destroy some of the more harmful constituents.

Dreyer's Diaplyte or Defatted T.B. Antigen.—In this preparation the lipoidal substances on which acid-fastness depends are removed from the bacilli. The bacilli are ground up with formalin and are then extracted with acetone in a Soxhlet apparatus at a temperature of 65°–70° C. for twenty to twenty-four hours; if the acid-fastness is not removed the extraction is repeated.

A number of other tuberculin preparations have been used, but the above are the most important.

Anti-tubercular Sera.—From what has been said regarding immunity reactions in tuberculosis it will be gathered that it is questionable whether the use of passive immunity in the treatment of tuberculosis has a rational basis. Several investigators, however, have introduced the sera of animals treated with the products of

tubercle bacilli for therapeutic purposes. Amongst these are Maragliano, who has treated dogs, asses, and horses with materials derived from the tubercle bacillus, and administers their serum in doses of 2 c.c. every two days in human tuberculosis. An anti-tubercular serum has also been introduced by Marmorek, who grows the bacilli in media unfavourable to their vitality and employs such growths for immunising animals whose serum he states is suitable for the treatment of human cases.

Methods of Examination.—(1) *Microscopic Examination.*—Tuberculosis is one of the comparatively few diseases in which a diagnosis can usually be definitely made by microscopic examination alone. In the case of sputum, one of the yellowish fragments which are often present ought to be selected; dried films are then prepared in the usual way, and stained by the Ziehl-Neelsen stain (p. 105). In the case of urine or other fluids, a deposit should first be obtained by centrifuging a quantity, or by allowing the fluids to stand in a tall glass vessel (an ordinary burette is very convenient). Film preparations are then made with the deposit and treated as before. If a negative result is obtained in a suspected case, repeated examination should be undertaken. To avoid risk of contamination with the smegma bacillus, the meatus of the urethra should be cleansed and the urine first passed should be rejected, or the urine may be drawn off with a sterile catheter. As stated above, it is only exceptionally that difficulty will arise to the experienced observer from this cause. (For points to be attended to, *vide* p. 331.) The detection of tubercle bacilli by microscopical methods in sputum, pus, fæces, and even tissues, has been greatly facilitated by the introduction of a preparation called "antiformin." This is a mixture of equal parts of liquor sodæ chlorinatæ (B.P.) and of a 15 per cent. solution of caustic soda. It has a remarkable disintegrative and dissolving action on the tissues, etc., so that after it has been allowed to act on sputum, for example, and the mixture is centrifuged, the resulting deposit is scanty and the tubercle bacilli, if present, are accordingly greatly concentrated. The time necessary may be judged of by the appearance of the mixture, but it will generally be found that the desired result will be obtained after about an hour if 1 part of sputum be added to 2 or 3 parts of 20 per cent. antiformin; the mixture should be shaken from time to time, especially when the sputum is tenacious.

(2) *Inoculation.*—The guinea-pig is the most suitable animal. If the material to be tested is a fluid, it is injected subcutaneously or into the peritoneum; if solid or semi-solid, it is placed in a small pocket in the skin, or it may be thoroughly broken up in sterile water or other fluid and the emulsion injected. By this method, material in which no tubercle bacilli can be found microscopically may sometimes be shown to be tubercular. Where other organisms are present, preliminary treatment with antiformin is advisable.

The following method of G. Haswell Wilson for treating tissues is to be recommended: The tissue to be investigated is cut up into small pieces with scissors, and is thoroughly rubbed up in a mortar with a small amount of dry sterile quartz sand. The rubbing up is continued till the fibrous tissue is disintegrated as far as possible, and the material forms a slightly moist, crumbling mass. The contents of the mortar are then washed into a wide test-tube with 15 to 20 c.c. of sterile saline solution. The sand is allowed to

sediment for a few minutes ; as it falls, it carries down with it any coarser particles of tissue which remain. The supernatant fine suspension is then pipetted off, and thoroughly mixed with an equal volume of 15 per cent. antiformin. After five minutes, during which it should be stirred continuously, the mixture is centrifuged at high speed for a few minutes, and the supernatant fluid is discarded. The sediment is shaken up with sterile saline solution and again centrifuged. The shaking up of the sediment with sterile saline solution and centrifuging are carried out three times in all, so that no trace of antiformin remains. The sediment resulting from the final centrifuging is used for making cultures (see below), or, after emulsifying with a convenient amount of sterile saline solution, is injected into a suitable animal.

(3) *Cultivation.*—The surest method of obtaining pure cultures is to produce tuberculosis in a guinea-pig by inoculation with tubercular material, and then, killing the animal after four or five weeks, to inoculate tubes of solidified blood serum or egg medium, under strict aseptic precautions, with portions of a tubercular organ, *e.g.* the spleen. The portions of tissue should be fairly large, and should be well rubbed into the broken surface of the medium. Cultures may, however, be obtained from sputum or tissues after treatment by antiformin as described in paragraph (2). If, then, inoculations be made from the deposit on blood serum or on Dorset's egg medium and glycerin egg medium, pure cultures of the tubercle bacillus may be obtained. The method is one which gives good results.

Petroff's method is also recommended as giving satisfactory results. In this, sputum is shaken with an equal volume of 3 per cent. caustic soda solution, and the mixture is placed for half an hour in the incubator at 37° C. At the end of this time it is made neutral to litmus with hydrochloric acid and then centrifuged. Some of the deposit thus obtained is then planted on egg medium to which crystal-violet has been added in the proportion of 1 : 10,000, the dye having an inhibitory action on the growth of various organisms. A pure culture of the tubercle bacillus is often obtained.

Twort has recommended exposing portions of sputum to the action of a 2 per cent. solution of ericolin (a glucoside) for an hour at 38° C. ; thereafter cultures are made on Dorset's medium.

(4) *Reactive phenomena.*—The presence of immune-substances in the blood and the tuberculin reaction, along with the methods of applying the respective tests, have been described above (p. 335).

CHAPTER XI

LEPROSY

LEPROSY is a disease of great interest, alike in its clinical and pathological aspects; whilst from the bacteriological point of view, also, it presents some striking peculiarities. The disease has a very wide geographical distribution. It occurs in certain parts of Europe—Norway, Russia, Greece, etc.—but is commonest in Asia, occurring in India, Syria, Persia, China, etc. It is prevalent in Africa, in the Pacific Islands, in certain parts of North and South America. In all these various regions the disease presents the same general features, and the study of its pathological and bacteriological characters, wherever such has been carried on, has yielded similar results.

Pathological Changes.—Leprosy is essentially a chronic disease, in which there is a great amount of tissue change without there being necessarily impairment of the general health. In other words, the local effects of the bacilli are well marked, often extreme, whilst the toxic phenomena are proportionately at a minimum unless during exacerbations.

There are two chief forms of leprosy. The one, usually called the nodular or tubercular form—*lepra tuberosa* or *tuberculosa*—is characterised by the growth of granulation tissue in a nodular form, or as a diffuse infiltration in the skin, in mucous membranes, etc., great disfigurement often resulting. In the other form, the anæsthetic—*maculo-anæsthetic* of Hansen and Looft—the outstanding changes are in the nerves, with consequent anæsthesia, paralysis of muscles, and trophic disturbances.

In the *nodular* form, the disease usually starts with the appearance of erythematous patches attended by fever, and these are followed by the development of small nodular thickenings in the skin, especially of the face, of the backs of hands and feet, and of the extensor aspects of arms and legs. These nodules enlarge and produce great distortion of the surface, so that, in the case of the face, an appearance is produced which has been described as "leonine." The thickenings occur chiefly in the cutis (Fig. 84), to a less extent in the subcutaneous tissue. The

epithelium often becomes stretched over them, and an oozing surface becomes developed, or actual ulceration may occur. The cornea and other parts of the eye, the mucous membrane of the mouth, nose, pharynx, and larynx may be the seat of similar nodular growths. Nodules in mucous membranes readily ulcerate, and in the nasal secretion large numbers of leprosy bacilli may be demonstrated; this is utilised for diagnostic purposes (*vide infra*). Internal organs, especially the

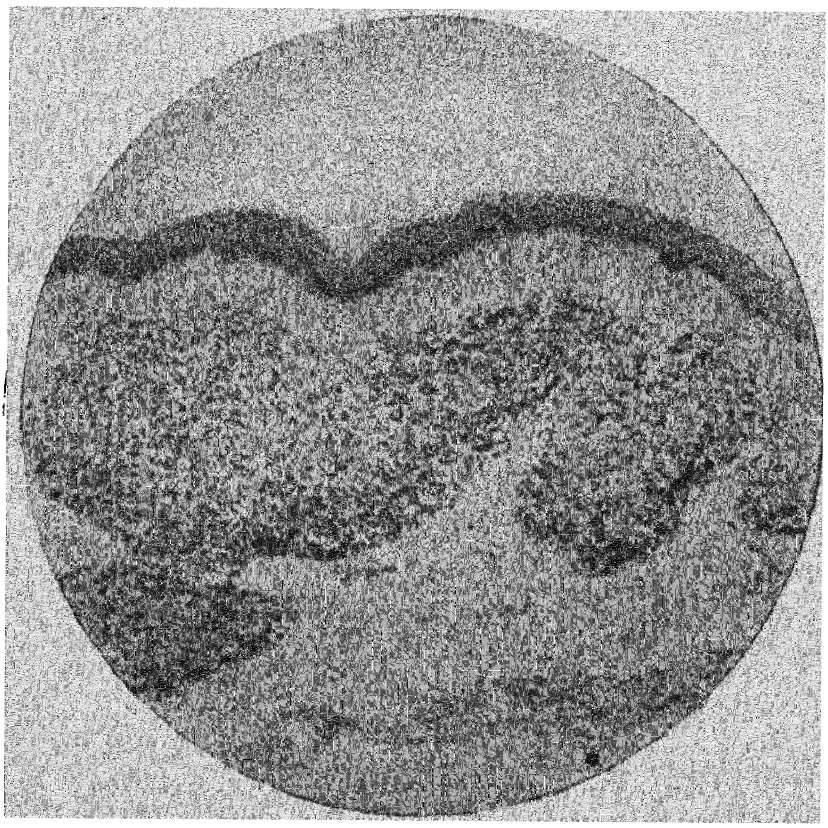


FIG. 84.—Section through leprous skin, showing the masses of cellular granulation tissue in the cutis; the dark points are cells containing bacilli deeply stained.
Paraffin section; Ziehl-Neelsen stain. ×80.

spleen, liver, and testicles, may become secondarily affected. In all situations the change is of the same nature, consisting in an abundant formation of granulation tissue, nodular or diffuse in its arrangement. In this tissue a large proportion of the cells are of rounded or oval shape, like hyaline leucocytes; a number of these may be of comparatively large size, and may show vacuolation of their protoplasm and a vesicular type of nucleus. These are often known as "lepra cells." Amongst the cellular elements there is a varying amount of stroma, which in the

earlier lesions is scanty and delicate, but in the older lesions may be very dense. Periarteritis is a common change, and very frequently the superficial nerves become involved in the nodules, and undergo atrophy. The tissue in the leprous lesions is comparatively vascular, at least when young, and, unlike tubercular lesions, never shows caseation. Some of the lepra cells may contain several nuclei, but we do not meet with cells resembling in their appearance tubercle giant-cells, nor does a focal arrangement like that in tubercle follicles occur.

In the *maculo-anæsthetic* form, the lesion of the nerves is the outstanding feature. These are the seat of diffuse infiltrations, which lead to the destruction of the nerve fibres. In the earlier stages, in which the chief symptoms are pains along the nerves, there occur patches on the skin (*maculæ*), often of considerable size, the margins of which show a somewhat livid congestion. Later, these patches become pale in the central parts, and the periphery becomes pigmented. There then follows a remarkable series of trophic disturbances, in which the skin, muscles, and bones are especially involved. The skin often becomes atrophied, parchment-like, and anæsthetic ; frequently pemphigoid bullæ or other skin eruptions occur. Partly owing to injury to which the feet and hands are liable from their anæsthetic condition, and partly owing to trophic disturbances, necrosis and separation of parts are liable to occur. In this way great distortion or mutilation results. The lesions in the nerves are of the same nature as those described above, but the granulation tissue is scantier, and has a greater tendency to undergo cicatricial contraction. This is to be associated with the fact that the bacilli are present in fewer numbers.

Bacillus of Leprosy (*Mycobacterium lepræ*).—This bacillus was first observed in leprous tissues by Hansen in 1871, and was the subject of several communications by him in 1874 and later. Further researches, first by Neisser in 1879, and afterwards by observers in various parts of the world, agreed in their main results, and confirmed the accuracy of Hansen's observations. The bacilli, as seen in scrapings of ulcerated leprous nodules, or in sections, have the following characters : They are thin rods of practically the same size as tubercle bacilli, which they also resemble both in appearance and in staining reaction. They are straight or slightly curved, and usually occur in groups, though single forms and pairs may be noted. When stained they may have a uniform appearance, or the protoplasm may be beaded like the tubercle bacillus. The beading is often of a " coarse " type. They often appear tapered at one or both extremities ;

occasionally there is slight club-like swelling. Degenerated and partially broken-down forms are also seen. They take up the basic aniline stains more readily than tubercle bacilli, but in order to stain them deeply, a powerful stain, such as carbol-fuchsin, is necessary. When stained, they resist decolorising, though they are more easily decolorised than tubercle bacilli (p. 109); variations, however, exist in this respect, some bacilli

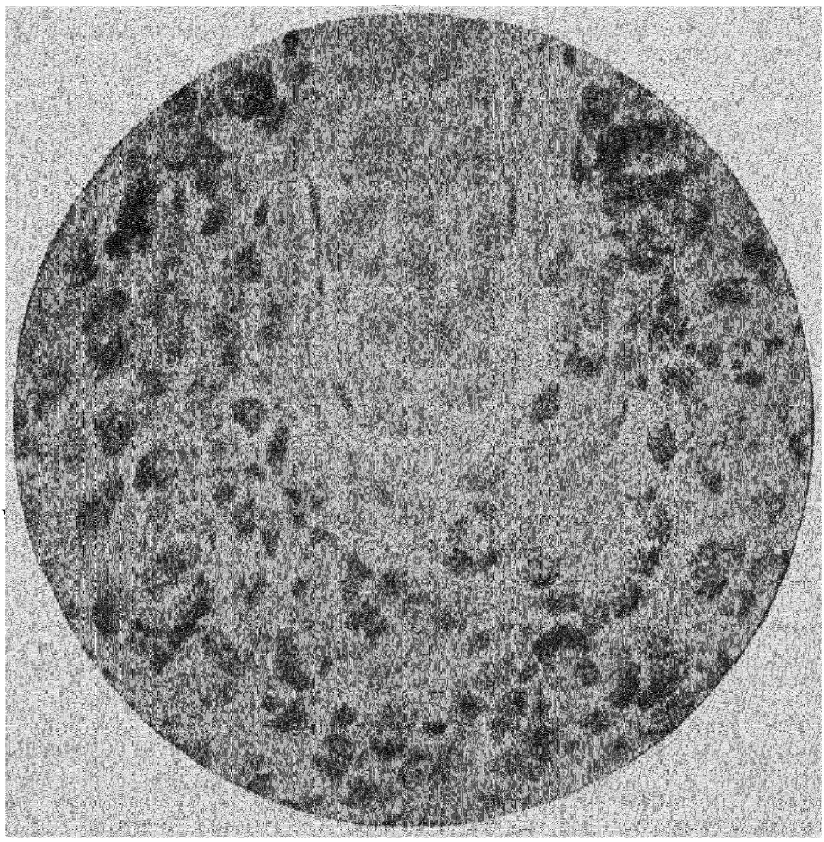


FIG. 85.—Superficial part of leprous skin; the cells of the granulation tissue appear as dark patches, owing to the deeply stained bacilli in their interior. In the upper part a process of epithelium is seen. Paraffin section; stained with carbol-fuchsin and Bismarck-brown. ×500.

losing the stain more readily than others. The bacilli are also readily stained by Gram's method. They are regarded as non-motile and non-sporing organisms.

Position of the Bacilli.—They occur in enormous numbers in the leprous lesions, especially in the nodular form—in fact, so numerous are they that the granulation tissue in sections, properly stained as above, presents quite a red colour under a low power of the microscope (Plate II., Fig. 8). The bacilli occur for the most part within the protoplasm of the round

cells of the granulation tissue, and are often so numerous that the structure of the cells is quite obscured (Fig. 85). They are often arranged in bundles which contain several bacilli lying parallel to one another, though the bundles lie in various directions (Fig. 86 and Plate II., Fig. 9). The appearance thus presented by the cells filled with bacilli is very characteristic. Bacilli are also found free in the lymphatic spaces, but the greater

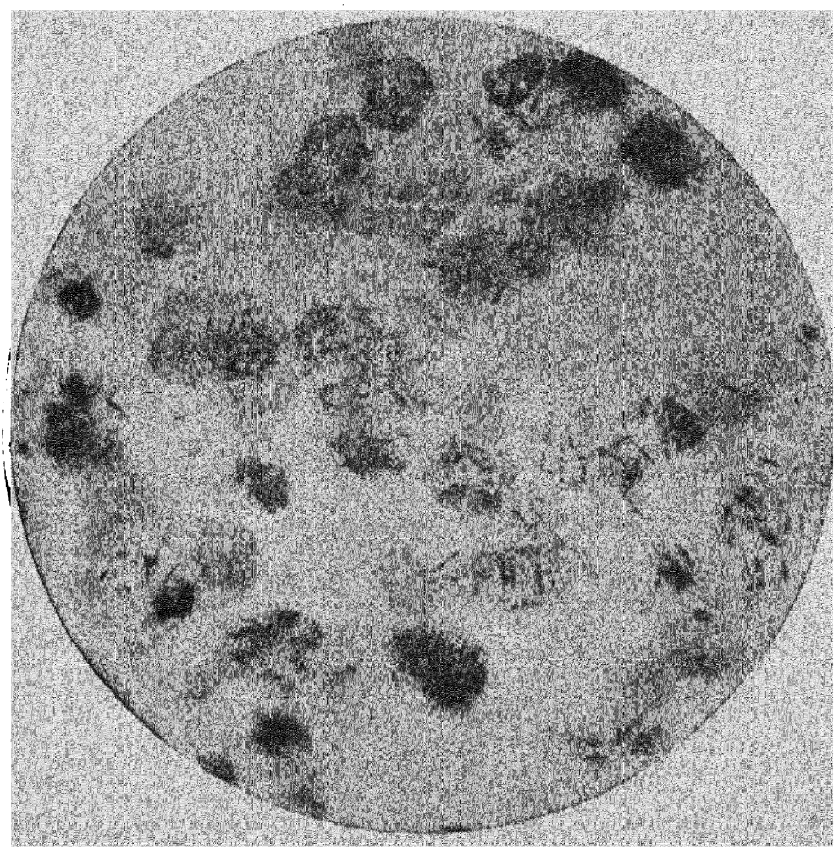


FIG. 86.—High-power view of portion of leprous nodule, showing the arrangement of the bacilli within the cells of the granulation tissue.

Paraffin section; stained with carbol-fuchsin and methylene-blue. ×1100.

number are undoubtedly contained within the cells. They are also found in spindle-shaped connective-tissue cells, in endothelial cells, and in the walls of blood vessels. They have been observed in a venous thrombus. They are for the most part confined to the connective tissue, but a few may be seen in the hair follicles and glands of the skin. Occasionally one or two may be found in the surface epithelium, where they probably have been carried by leucocytes, but this position is, on the whole, exceptional. They occur also in large numbers in the

lymphatic glands associated with the affected parts. In the internal organs—liver, spleen, etc.—when leprous lesions are present, the bacilli are also found, though in relatively smaller numbers. In the nerves in the anæsthetic form they are less numerous than in the nodular lesions, and in the sclerosed parts it may be impossible to find any. They are absent from the *maculæ*, which are essentially trophic lesions.

Their spread is chiefly by the lymphatics, though distribution by the blood stream also occurs. They are said to have been found in the blood during the presence of fever and the eruption of fresh nodules, and they have also been observed in the blood vessels *post mortem*, chiefly contained within leucocytes. A few may be detected in some cases in various organs which show no structural change, especially in the capillaries. The brain and spinal cord are almost exempt, but in some cases bacilli have been found even within nerve cells.

Cultivation.—Attempts to cultivate the bacillus have in the majority of cases led to negative results, and the positive findings recorded lack the necessary confirmation. Further, it seems not unlikely that the acid-fast bacilli " cultivated " from leprosy represent saprophytic non-pathogenic organisms which are widely distributed and may occur on the skin We have examined a number of the reputed " B lepræ " strains, and, like others, have found them to resemble closely these non-pathogenic types in their biological characters Kedrowski cultivated an organism which in culture appeared as a non-acid-fast diphtheroid, but which regained the acid-fast character in the tissues of animals. When injected into mice and rats it produced, in a certain proportion of cases, lesions which presented the essential features of human leprosy, the bacilli occurring in large numbers within rounded cells. This organism grew very slowly and produced an irregular whitish growth of moist appearance closely resembling that of the avian tubercle bacillus. Bayon confirmed Kedrowski's results. Clegg grew a small acid-fast bacillus on plain agar medium along with amœbæ and symbiotic bacteria, and then, by killing the contaminating organisms by means of heat, obtained a pure growth of a chromogenic acid-fast bacillus. Duval, following out this work, obtained confirmatory results, but in addition to Clegg's bacillus he cultivated a slowly growing non-chromogenic bacillus which only grew on special media. This latter he believed to be probably the causal organism. He laid stress especially upon its very slow growth, the colonies after eight to ten months being about the size of those of the influenza bacillus, and upon its requiring the presence of the products of protein digestion. Twort also claimed to have cultivated the organism on glycerin egg medium containing dead tubercle bacilli in the proportion of 1 per cent. Rost and Williams cultivated a pleomorphous organism, a streptothrix, which appeared in the form of bacilli or branched filaments, and both of these forms were acid-fast or non-acid-fast. Their organism, it is to be noted, however.

grew comparatively rapidly, growth being visible within a week, whilst in the case of the organisms of Kedrowski, Duval, and Twort, growth only appeared after several weeks. Furthermore, the organisms of Duval and Twort appeared only in the bacillary form, whilst those of the other observers mentioned show pleomorphism. Bayon compared the pathogenic properties of the bacilli cultivated by different workers, and found that only Kedrowski's bacillus and that cultivated by himself, which he regarded as the same organism, produced in animals lesions similar to those of leprosy, the cells in the lesions being packed with bacilli, and there appears to be no doubt that in his preparations a multiplication of the organism had taken place. He also found that only these two strains gave a distinct fixation of complement (p. 134) when tested with the serum of leprous patients. It is quite clear that certain of the organisms cultivated by various workers and claimed to be leprosy bacilli present essential differences, and some of them certainly correspond closely to saprophytic acid-fast bacillary types (*vide* p. 329). It is still impossible to express any definite opinion on the subject.

For a long time attempts to transmit leprosy to the lower animals were unsuccessful. The only exception to this statement is afforded by the experiments of Melcher and Orthmann, who produced nodules in the organs of rabbits after inoculating the anterior chamber of the eye with leprous material, the cells in the nodules containing numerous bacilli. These results have been generally called in question, but in view of recent work it is quite possible that the lesions were really of leprous nature. Sugai has found that the Japanese dancing-mouse is comparatively susceptible to inoculation with leprous material, and Duval has confirmed this observation. The experiments of Kedrowski and Bayon have already been referred to. It is to be noted, however, that in all these cases success was only obtained in a certain proportion of cases, and also that the picture of cells packed with bacilli has also been obtained by the injection of acid-fast saprophytes. In tuberculosis in mice a similar picture is obtained. It would accordingly be a mistake to place much reliance on this point. Experiments have also been performed on monkeys, but the results cannot be regarded as conclusive.

It is interesting to note that a disease occurs under natural conditions in rats which presents many points of close similarity to leprosy. It is very widespread, having been observed in Europe, Asia, America, and Australia ; an excellent description was given by G. Dean. In this affection there are lesions in the skin which resemble those in leprosy, and the cells contain enormous numbers of an acid-fast bacillus. The disease can be transmitted to rats by inoculation with the tissue juices

containing the bacilli, but not to animals of other species. The relations of this affection to human leprosy have not yet been established. Bayon claims to have cultivated the bacillus of rat leprosy, and finds that it is practically identical, as regards both cultural characters and pathogenic effects, with the organism obtained from the human disease.

It would also appear that leprosy is not readily inoculable in the human subject. In a well-known case described by Arning, a criminal in the Sandwich Islands was inoculated in several parts of the body with leprosy tissue. Two or three years later, well-marked nodular leprosy appeared, and led to a fatal result. This experiment, however, is open to the objection that the individual before inoculation had been exposed to infection in a natural way, having been frequently in contact with lepers. In other cases, inoculation experiments on healthy subjects and inoculations in other parts of leprous individuals have given negative results. It has been supposed by some that the failure to obtain cultures and to reproduce the disease experimentally may be partly due to the bacilli in the tissues being dead. The varying results of inoculation of the human subject present, to some extent, a parallel to the results of experiments on animals, as given above.

The facts stated with regard to cultivation and inoculation experiments serve to distinguish the leprosy bacillus all the more strongly from other organisms. It was supposed at one time that leprosy was a form of tubercle, or tubercle modified in some way, but for this there is no evidence. It should also be mentioned that tubercle is a not uncommon complication in leprous subjects, in which case it presents the ordinary characters. It has been found that a considerable proportion of lepers react to tuberculin like tubercular patients. This result has been variously interpreted, some considering that tuberculosis is also present in such cases, whilst others maintain that the reaction may be given in the absence of tubercle. If, as is probable, the latter is the case, the result most likely depends on the close relationship of the organisms of the two diseases ; it by no means proves their identity. Another curious fact is that the Wassermann reaction (p. 135) may be given by the serum of leprous patients (in about 50 per cent., according to some observers). It is uncertain whether the reaction in leprosy is independent of the concurrent presence of syphilis, as has been suggested by some observers. No method of treatment of leprosy has been supplied from the bacteriological side, but the injection of chaulmoogra oil has been found to exert a favourable therapeutic

effect, and has apparently led to cure in early cases. The ethyl esters to which the effect of the oil is attributed have been investigated by Sir Leonard Rogers.

The mode by which leprosy is transmitted has been the subject of great controversy. It has been considered to be a hereditary disease transmitted from a parent to the offspring. There appears to be no doubt, however, that on the one hand leprous subjects may bear children free from leprosy, and that on the other hand healthy individuals entering a leprous district may contract the disease. Of the latter occurrence there is the well-known instance of Father Damien, who contracted leprosy after going to the Sandwich Islands. In view of all the facts, there can be little doubt that leprosy is transmitted by direct contact in certain conditions, though its contagiousness is not of a high order; this is the presently accepted view. Close contact over a long period, such as occurs in families, is necessary for the spread of the disease. It has been suggested also that certain insects may be responsible for transmission of the disease, but there is no reliable evidence to support this idea.

Methods of Diagnosis.—Film preparations should be made with the discharge from any ulcerated nodule which may be present, or from the scraping of a portion of excised tissue, and should be stained as above described. The presence of large numbers of bacilli situated within the cells and giving the staining reaction of leprosy bacilli, is conclusive. It is more satisfactory, however, to make microscopic sections through a portion of the excised tissue, when the structure of the nodule and the arrangement of the bacilli can be readily studied. Films of the nasal secretion (*vide supra*) are also examined in the same way, and may yield positive results even in maculo-anæsthetic cases. The points of difference between leprosy and tubercle have already been stated, and in most cases there is really no difficulty in distinguishing the two conditions. A negative result, on inoculating a guinea-pig with the suspected material, will exclude tuberculosis.

CHAPTER XII

GLANDERS, MELIOIDOSIS, AND RHINOSCLEROMA

Glanders

The bacillus of glanders, B. mallei (*Pfeifferella mallei*), was discovered by Löffler and Schutz, the announcement of this discovery being made towards the end of 1882. They not only obtained pure cultures of this organism from the tissues in the disease, but by experiments on horses and other animals conclusively established its causal relationship. These have been fully confirmed. The same organism was also cultivated from the disease in the human subject, first by Weichselbaum in 1885, who obtained it from the pustules in a case of acute glanders in a woman, and by inoculation of animals obtained results similar to those of Löffler and Schutz.

Within more recent times a substance, *mallein*, has been obtained from the cultures of the glanders bacillus by a method similar to that by which tuberculin is prepared, and has been found to produce effects in animals suffering from glanders corresponding to those produced by tuberculin in tuberculous animals.

The Natural Disease.—Glanders chiefly affects the equine species—horses, mules, and asses. Horned cattle, on the other hand, are quite immune, whilst goats and sheep occupy an intermediate position, the former being rather more susceptible and occasionally suffering from the natural disease. It occurs also in some of the carnivora—cats, lions, and tigers in menageries, which animals are infected from the carcases of animals affected with the disease. Many of the small rodents are highly susceptible to experimental inoculation (*vide infra*).

Glanders is found also in man as the result of direct inoculation of some wound of the skin or other part by means of the discharges or diseased tissues of an animal affected, and hence is commonest amongst grooms and others whose work brings them into contact with horses; even amongst them it is a comparatively rare disease.

In horses the lesions are of two types, to which the names "glanders" proper and "farcy" have been given, though both may exist together. In glanders proper, the septum nasi and adjacent parts are chiefly affected, there occurring in the mucous membrane nodules which are at first firm and of somewhat translucent grey appearance. The growth of these is usually attended by inflammatory swelling and profuse catarrhal discharge. Afterwards the nodules soften in the centre, break down, and give rise to irregular ulcerations. Similar lesions, though in less degree, may be found in the respiratory passages. Associated with these lesions there is usually implication of the lymphatic glands in the neck, mediastinum, etc.; and there may be in the lungs, spleen, liver, etc., nodules of the size of a pea or larger, of greyish or yellow tint, firm or somewhat softened in the centre, and often surrounded by a congested zone. The term "farcy" is applied to the affection of the superficial lymphatic vessels and glands, which is specially seen where infection takes place through an abrasion of the skin, such as is often produced by the rubbing of the harness. The lymphatic vessels become irregularly thickened, so as to appear like knotted cords, and the associated lymphatic glands become enlarged and firm; suppurative softening usually follows, and there may be ulceration. These thickenings are often spoken of as "farcy buds" and "farcy pipes." In farcy, also, secondary nodules may occur in internal organs and the nasal mucous membrane. The disease is often present in a "latent form," and its presence can only be detected by the mallein test (*vide* p. 360). In the ass the disease runs a more acute course than in the horse.

In man the disease is met with in two forms, an acute and a chronic—though intermediate forms also occur, and chronic cases may take on the characters of the acute disease. The site of inoculation is usually on the hand or arm—by means of some scratch or abrasion, or possibly by infection along a hair follicle—sometimes on the face, and occasionally on the mucous membrane of the mouth, nose, or eye. In the *acute* form there appears at the site of inoculation inflammatory swelling, attended usually with spreading redness, and the lymphatics in relation to the part also become inflamed, the appearances being those of a "poisoned wound." These local changes are soon followed by marked constitutional disturbance, and by a local or widespread eruption on the surface of the body, at first papular and afterwards pustular, and later there may form in the subcutaneous tissue and muscles larger masses which soften and suppurate, the pus being often mixed with blood; suppuration may occur also in the joints. In some cases the nasal mucous membrane may be secondarily infected, and thence inflammatory swelling may spread to the tissues of the face. The patient usually dies in two or three weeks, sometimes sooner, with the symptoms of pyæmia. In addition to the lesions mentioned, there may

be foci, usually suppurative, in the lungs (attended often with pneumonic consolidation), in the spleen, liver, bone-marrow, salivary glands, etc. In the *chronic* form a local granulomatous condition may occur, which usually breaks down and gives rise to the formation of an irregular ulcer with thickened margins, and sanious, often foul, discharge. The ulceration spreads deeply as well as superficially, and the thickened lymphatics also have a great tendency to ulcerate, though the lymphatic system is not so prominently affected as in the horse. Deposits may form in the subcutaneous tissue and muscles, and the mucous membrane may become affected. The disease may run a very chronic course, lasting for months or even years, and recovery may occur; on the other hand, such a case may at any time take on the characters of the acute form of the disease and rapidly become fatal. Even when there is apparent recovery recurrence may occur.

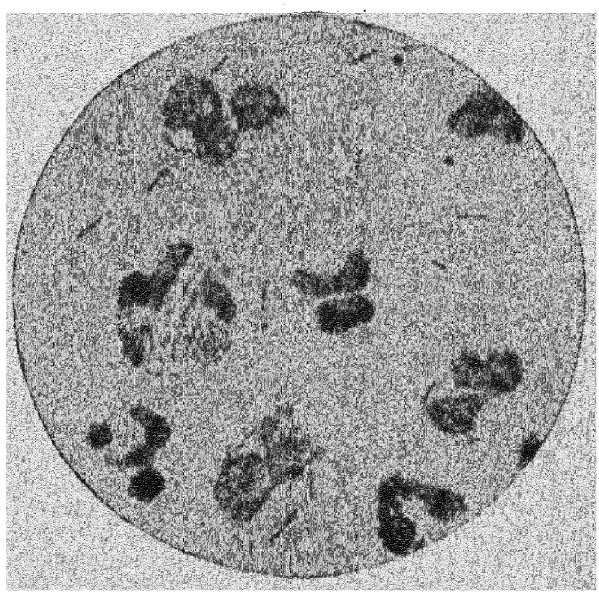


FIG. 87.—Glanders bacilli—several contained within leucocytes—from peritoneal exudate in a guinea-pig. Stained with weak carbol-fuchsin. ×1000.

The Glanders Bacillus.—*Microscopical Characters.*—The glanders bacilli are minute rods, straight or slightly curved, with rounded ends, and about the same length as tubercle bacilli, but rather thicker (Fig. 87). They show, however, considerable variations in size and in appearance, and their protoplasm is often broken up into a number of deeply stained portions with unstained intervals between. These characters are seen both in the tissues and in cultures, but, as in the case of many organisms, irregularities in form and size are more pronounced in cultures (Fig. 88); short filamentous forms 8 to 12 μ in length are sometimes met with, but these are on the whole rare. The organism is non-motile and does not form spores.

In the tissues the bacilli usually occur irregularly scattered amongst the cellular elements; a few may be contained within leucocytes and connective-tissue corpuscles, but the position of

most is extra-cellular. They are most abundant in the acute lesions, in which they may be found in considerable numbers; but in the chronic nodules, especially when softening has taken place, they are few in number, and it may be impossible to find any in sections.

Staining.—The glanders bacillus stains with simple watery solutions of the basic stains, but somewhat faintly (better when an alkali or a mordant, such as carbolic acid, is added), and even when deeply stained it readily loses the colour when a decolorising agent such as alcohol is applied. It is Gram-negative. In film preparations from fresh glanders nodules the bacilli can be readily found by staining with any of the ordinary combinations, *e.g.* carbol-thionin-blue or weak carbol-fuchsin. In the case of sections, we have obtained the best results by carbol-thionin-blue, and we prefer to dehydrate by the aniline-oil method (p. 101).

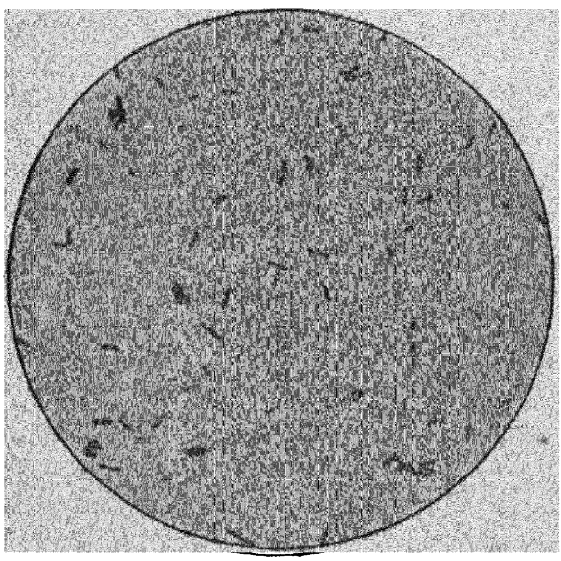


FIG. 88.—Glanders bacilli, from a pure culture on glycerin agar. Stained with carbol-fuchsin and partially decolorised to show segmentation of protoplasm. ×1000.

McFadyean recommends that after sections have been stained in Löffler's methylene-blue and slightly decolorised in weak acetic acid, they should be treated for fifteen minutes with a saturated solution of tannic acid; thereafter they are washed thoroughly in water, and as a contrast-stain a 1 per cent. solution of acid fuchsin may be applied for half a minute; they are then dehydrated, cleared, and mounted.

Cultivation.—(For the methods of separation, *vide infra.*) The glanders bacillus grows readily on most of the ordinary media, but a somewhat high temperature is necessary, growth taking place most rapidly at 35°–37° C. Though a certain amount of growth occurs down to 21° C., a temperature above 25° C. is always desirable.

On *agar* and *glycerin agar,* in stroke cultures, growth appears along the line as a uniform streak of greyish-white colour and somewhat transparent appearance, with moist-looking surface,

and when touched with a needle is found to be of rather slimy consistence. Later it spreads laterally for some distance, and the layer becomes of slightly brownish tint.

On *serum media* the growth is somewhat similar but more transparent, the separate colonies being in the form of round and almost clear drops. In subcultures at the body temperature growth is visible within twenty-four hours, but when fresh cultures are made from the tissues it may not be visible till the second day. Serum or potato is much more suitable for cultivating from the tissues than the agar media.

In *bouillon,* growth forms at first a uniform turbidity, but soon settles to the bottom, and after a few days forms a pretty thick flocculent deposit of slimy and somewhat tenacious consistence.

On *potato* (provided the reaction is not too acid), at 30°–37°C. the glanders bacillus flourishes well and produces a characteristic appearance, incubation at the higher temperature, however, being advisable. Growth proceeds rapidly, and on the third day has usually formed a transparent layer of slightly yellowish tint, like clear honey in appearance. On subsequent days, the growth still extends and becomes darker in colour and more opaque, till about the eighth day it has a reddish-brown or chocolate tint. The characters of the growth on potato, along with the microscopical appearances, are quite sufficient to distinguish the glanders bacillus from every other known organism (sometimes the cholera organism and the B. pyocyaneus produce a somewhat similar appearance, but they can be readily distinguished by their other characters). Potato is also a suitable medium for starting cultures from the tissues; in this case minute transparent colonies become visible on the third day, and afterwards present the appearances just described.

Powers of Resistance.—The glanders bacillus is not killed at once by drying, but usually loses its vitality after fourteen days in the dry condition, though sometimes it lives longer. It is not quickly destroyed by putrefaction, as it has been found to be still active after remaining two or three weeks in putrefying fluids. It has comparatively feeble resistance to heat and antiseptics. Löffler found that it was killed in ten minutes in a fluid kept at 55° C., and in from two to three minutes by a 5 per cent. solution of carbolic acid.

Experimental Inoculation.—In horses, subcutaneous injection of the glanders bacillus in pure culture reproduces all the important features of the disease. This fact was established at a comparatively early date by Löffler and Schutz, who, after one doubtful experiment, successfully inoculated two horses in this

way, the cultures used having been grown for several generations outside the body. The ass is even more susceptible than the horse, the disease in the former running a more rapid course, but with similar lesions. The ass can be readily infected by simple scarification and inoculation with glanders secretion, etc. (Nocard).

Of small animals, field-mice and guinea-pigs are the most susceptible; on the other hand, house-mice and white mice enjoy an almost complete immunity. In field-mice, subcutaneous inoculation is followed by a very rapid disease, usually leading to death within eight days, the organisms becoming generalised and producing numerous minute nodules, especially in the spleen, lungs, and liver. In the guinea-pig the disease is less acute. At the site of inoculation an inflammatory swelling forms, which soon softens and breaks down, leading to the formation of an irregular crateriform ulcer with indurated margins. The lymphatic vessels become infiltrated, and the corresponding lymphatic glands become enlarged to the size of peas or small nuts, softened, and semi-purulent. The animal sometimes dies in two or three weeks, sometimes not till later. Secondary nodules, in varying numbers in different cases, may be present in the spleen, lungs, bones, nasal mucous membrane, testicles, etc.; in some cases a few nodules are found in the spleen alone. Intraperitoneal injection in the male guinea-pig is followed, as pointed out by Straus, by a very rapid and semi-purulent affection of the tunica vaginalis, shown during life by great swelling and redness of the testicles, which changes may be noticeable in two or three days, or even earlier. This method of inoculation has been found of service for purposes of cultivation and diagnosis. Rabbits are less susceptible than guinea-pigs, and the effect of subcutaneous inoculation is somewhat uncertain. Accidental inoculation of the human subject with pure cultures of the bacillus has in more than one instance been followed by the acute form of the disease and a fatal result.

Mayer found that when the glanders bacillus was injected along with melted butter into the peritoneum of a guinea-pig, it showed filamentous, branching, and club-shaped forms; in other words, it presented the characters of a streptothrix. Lubarsch, on the other hand, in a comparative study of the results of inoculation with acid-fast and other bacilli, found none of the above characters in the case of the glanders bacillus.

Action on the Tissues.—The glanders bacillus causes a more rapid and more marked inflammatory reaction than the tubercle bacillus; there is more leucocytic infiltration and less proliferative

change. Thus the centre of an early glanders nodule shows a dense aggregation of leucocytes, most of which are polymorphonuclear, whilst in the central parts many show fragmentation of nuclei with the formation of numerous chromatin granules. And further, the inflammatory change may be followed by suppurative softening of the tissue, especially in certain situations, such as the subcutaneous tissue and lymphatic glands. The nodules, therefore, in glanders, as Baumgarten put it, occupy an intermediate position between miliary abscesses and tubercles. The diffuse coagulative necrosis and caseation which are so common in tubercle do not occur to the same degree in glanders, and typical giant-cells are not formed. The tendency to spread by the lymphatics is always a well-marked feature. and when the bacilli gain entrance to the blood stream they soon settle in the various tissues and organs. Accordingly, even in acute cases it is usually quite impossible to detect the bacilli in the circulating blood, though sometimes they have been found. It is an interesting fact, shown by observations of the disease both in the human subject and in the horse, as well as by experiments on guinea-pigs, that the mucous membrane of the nose may become infected by means of the blood stream—another example of the tendency of organisms to settle in special sites.

Mode of Spread.—Glanders usually spreads from a diseased animal by direct contagion with the discharge from the nose or from the sores, etc. There is no evidence that in man the disease is produced by inhalation of the bacilli in the dried condition. Some authorities consider that pulmonary glanders may be produced in this way in the horse, whilst others maintain that in all cases there is first a lesion of the nasal mucous membrane or of the skin surface, and that the lung is affected secondarily. Babés, however, found that the disease could be readily produced in susceptible animals by exposing them to an atmosphere in which cultures of the bacillus had been pulverised. He also found that inunction of the skin with vaseline containing the bacilli might produce the disease, the bacilli in this case entering along the hair follicles.

Serum Reactions.—Shortly after the discovery of agglutination in typhoid fever, McFadyean found that the serum of glandered horses possessed the power of agglutinating glanders bacilli. His later observations showed that in the great majority of cases of glanders a 1 : 50 dilution of the serum produces marked agglutination in a few minutes, whilst in the great majority of non-glandered animals no effect is produced under these conditions. The test performed

in the ordinary way is, however, not absolutely reliable, as exceptions occasionally occur in both directions, *i.e.* negative results by glandered animals and positive results by non-glandered animals. He found that a more delicate and reliable method is to grow the bacillus in bouillon containing a small proportion of the serum to be tested. In this way he obtained a distinct sedimenting reaction with a serum which did not agglutinate at all distinctly in the ordinary method. Within recent times the sedimentation test by the ordinary method (p. 125) has been most generally used. The general result seems to be that distinct sedimentation within thirty-six hours with a serum dilution of 1 : 1000 may be taken as a positive result, indicating the presence of glanders ; whilst reactions with dilutions between this and 1 : 500 are highly suspicious but not conclusive. The fixation of complement test (p. 134) is also applicable in the case of glanders, and this has given valuable results in the hands of various observers. Precipitin reactions may also be obtained on the addition of mallein or an extract of the glanders bacillus to the serum of a glandered animal. These reactions, which of course depend on the presence of antibodies in the blood in glanders, form important auxiliaries to the method of diagnosis by means of mallein.

Mallein and its Preparation.—Mallein is obtained from cultures of the glanders bacillus grown for a suitable length of time, and, like tuberculin, is really a mixture comprising (1) substances in the bodies of the bacilli, and (2) their soluble products, not destroyed by heat, along with substances derived from the medium of growth. It was at first obtained from cultures on solid media by extracting with glycerin or water, but is now usually prepared from cultures in glycerin bouillon. Such a culture, after being allowed to grow for three or four weeks, is sterilised by heat either in the autoclave at 115° C. or by steaming at 100° C. It is then filtered through a Chamberland filter. The filtrate constitutes fluid mallein. Usually a little carbolic acid (0·5 per cent.) is added as a preservative. Of such mallein 1 c c. is usually the dose for a horse (McFadyean). Foth has prepared a dry form of mallein by throwing the filtrate of a broth culture, evaporated to one-tenth of its bulk, into twenty-five or thirty times its volume of alcohol. A white precipitate is formed, which is dried over calcium chloride and then under an air-pump. A dose of this dry mallein is 0·05 to 0·07 gram.

The Use of Mallein as a Means of Diagnosis.—In using mallein for the diagnosis of glanders, the temperature of the animal ought to be observed for some hours beforehand, and after subcutaneous injection of a suitable dose, it is taken at definite intervals—according to McFadyean at the sixth, tenth, fourteenth, and eighteenth hours afterwards, and on the next day. Here both the local reaction and the temperature are of importance. In a glandered animal, at the site of inoculation there is a somewhat tender local swelling, which reaches a diameter of 5 inches at least, the maximum size not being attained until twenty-four hours afterwards. The temperature rises 1·5° to 2° C., or more, the maximum generally occurring in from eight to sixteen hours. If the temperature never rises as much as 1·5°, the reaction is considered doubtful. In the negative reaction given by an animal free from glanders, the rise of temperature does not usually exceed 1°, the local swelling reaches the diameter of 3 inches at most, and has much diminished at the

end of twenty-four hours. In the case of dry mallein, local reaction is less marked. Veterinary authorities are practically unanimous as to the great value of the mallein test as a means of diagnosis. It has recently been shown that mallein instilled into the conjunctival sac, or inoculated by scarification into the skin of glandered animals, gives corresponding reactions to the ophthalmic and cutaneous tuberculin reactions in cases of tuberculosis (p. 336); in the case of glanders the conjunctival reaction would appear to be the more convenient and reliable. By the use of the mallein test followed by slaughter of the animals thereby found to be infected glanders has been practically abolished in this country.

Methods of Examination.—Microscopic examination in a case of suspected glanders will at most reveal the presence of bacilli corresponding in their characters to the glanders bacillus. An absolute diagnosis cannot be made by this method. Cultures may be obtained by making successive strokes on blood serum or on potato, and incubating at 37° C. The colonies of the glanders bacillus do not appear till two or three days afterwards. This method may fail unless a considerable number of the glanders bacilli are present. The most certain method, however, is by inoculation of a guinea-pig, either by subcutaneous or intra-peritoneal injection. By the latter method, as above described, lesions are much more rapidly produced, and are more characteristic. If, however, there have been other organisms present, the animal may die of a septic peritonitis, though even in such a case the glanders bacilli will be found to be more numerous in the tunica vaginalis, and may be cultivated from this situation. It is extremely doubtful whether the application of mallein in diagnosis of the disease in the human subject is justifiable. There is a certain risk that it may lead to the lesions assuming a more acute character; moreover, culture and inoculation tests are generally available. In the case of horses, etc., a diagnosis will, however, be much more easily and rapidly effected by means of mallein, or by one of the serum reactions described above. In some cases of acute glanders in the human subject the bacillus has been obtained in cultures from the blood during life.

Melioidosis.—This disease, which resembles glanders closely, was first observed by Whitmore in Rangoon, and its main features and the causal organism were described by him in 1913. He cultivated the latter from 38 cases and gave an account of its characters; the organism is now known as B. whitmori. The disease has also been found to occur by Stanton and Fletcher in the Malay States, and they have shown that it is naturally an epizootic among small rodents—rabbits, guinea-pigs, and rats—from which it is communicable to man. The clinical features of the disease in the human subject are very similar to those of glanders, though it tends

to be, on the whole, rather more acute than the latter. The pathological changes likewise are of similar nature, a prominent feature being the occurrence of caseous or semi-purulent areas in the lungs with marked congestion around them ; the histological changes also have been found to be practically identical in the two diseases. B. whitmori is a small bacillus which, in its morphological characters and in its staining reactions, resembles the B. mallei. It differs from the latter, however, in being actively motile, in growing on gelatin at 20° C. in which it produces liquefaction, and in forming a pellicle in bouillon. The growth on agar media is of two types, a corrugated and a mucoid, and strains which are of the latter type produce on potato a growth similar to that of the glanders bacillus. Susceptible animals can be infected by scarification, by feeding, or by the simple application of cultures to the nasal mucosa. A characteristic feature in the infected animals is a discharge from the nose and eyes, and *post mortem* numerous small nodules are found in the internal organs. Apparently the disease does not occur in the horse, and Stanton and Fletcher have found that this animal is insusceptible to inoculation. These observers have found that the B. whitmori is serologically closely allied to the B. mallei; in fact, a strain of the latter gave almost similar reactions. They found that a case of chronic human melioidosis gave a positive mallein reaction, and that the serum agglutinated certain strains of B. mallei. From this short account it will be seen that while melioidosis and glanders closely resemble one another, they present also interesting points of difference.

Rhinoscleroma

This disease is considered here as, from the anatomical changes, it also belongs to the group of infective granulomata. It is characterised by the occurrence of chronic nodular thickenings in the skin or mucous membrane of the nose, or in the mucous membrane of the pharnyx, larynx, or upper part of the trachea. The nodules are of considerable size, sometimes as large as a pea ; in the earlier stages they are comparatively smooth on the surface, but later they become shrunken and the centre is often retracted. The disease is scarcely ever met with in this country, but is of not uncommon occurrence on the Continent, especially in Austria and Poland. In the granulation tissue of the nodules there are to be found numerous round and rather large cells, which have peculiar characters and are often known as the cells of Mikulicz. Their protoplasm contains a collection of somewhat gelatinous material which may fill the cell and push the nucleus to the side. Within these cells there is present a characteristic bacillus, occurring in little clumps or masses, chiefly in the gelatinous material. A few bacilli also lie free in the lymphatic spaces around. This organism was first observed by Frisch, and is now known as the bacillus of rhinoscleroma (*Encapsulatus rhinoscleromatis*). The bacilli have the form of short oval rods, which, when lying separately, can be seen to possess a distinct capsule ; in all their microscopical characters they correspond closely with Friedländer's pneumobacillus. They are Gram-negative, and as a rule they are the only organisms demonstrable.

From the affected tissues this bacillus can be easily cultivated by

the ordinary methods. In the characters of its growth in the various culture media it presents a close similarity to the pneumobacillus, as it does also in its fermentative action in milk and sugar-containing fluids. Minor differences have been described, but it is doubtful whether any distinct line of difference can be drawn between the two organisms so far as their microscopical and cultural characters are concerned.

The serum of patients suffering from the disease gives fixation of complement when tested with an emulsion of the bacillus, but varying results have been obtained as regards the validity of the test in the differentiation of the bacillus from the allied organisms.

The evidence that the organisms described are the cause of this disease consists essentially in their constant presence and their special relation to the affected tissues, as already described. Experimental inoculation has thrown little light on the subject, though one observer has described the production of nodules on the conjunctivæ of guinea-pigs The relation of the rhinoscleroma organism to that of Friedländer is still a matter of doubt, and the matter has been further complicated by the fact that a bacillus possessing closely similar characters has been found to be very frequently present in ozæna, and is often known as the *Bacillus ozænæ*. The last-mentioned organism is said to have more active fermentative powers. From what has been stated it will be seen that a number of organisms, closely allied in their morphological characters, have been found in the nasal cavity in healthy or diseased conditions. There is no doubt that rhinoscleroma is a specific disease with well-marked characters, and it is quite possible that one member of this group of organisms may be the causal agent, though indistinguishable from others by culture tests. There is, however, a tendency on the part of recent investigators, *e.g* Perkins, to consider the "bacillus of rhinoscleroma" to be identical with the pneumobacillus, and its presence in the affected tissues to represent merely a secondary invasion. The subject is one on which more light is still required.

CHAPTER XIII

ACTINOMYCOSIS AND ALLIED DISEASES

ACTINOMYCOSIS is the most important of the group of diseases produced by organisms of the *streptothrix* group. It occurs in man in common with certain of the domestic animals, though it is more frequent in the latter, especially in oxen, swine, and horses. The parasite was first discovered in the ox by Bollinger, and described by him in 1877. The name *Actinomyces* or *Ray fungus* was applied to it by the botanist Harz, owing to the radiate club-shaped structures characteristic of colonies of the parasite in the tissues. In 1878 Israel described the parasite in the human subject. It is, however, to be noted that the term "actinomyces," as originally used, does not represent one parasite but a number of allied species, as cultures obtained from various sources have presented considerable differences. Moreover, it has been found by Lignières and Spitz that in a common type of actinomycosis in the ox the colonies are formed not by a streptothrix but by a bacillus to which they have given the name of *Actinobacillus*. The term "actinomycosis" accordingly does not represent a specific disease, but may conveniently be retained for infections in which the parasite forms "granules" or colonies composed of a mycelium or meshwork of a filamentous organism or exhibiting radial club-shaped bodies.[1] Such infections have now been shown to be of comparatively common occurrence. Further, other distinct species of streptothrix, without the characteristic arrangement, have been cultivated from isolated cases of disease in the human subject where the lesions resemble more or less closely those of actinomycosis. In one or two instances the organism has been found to be "acid-fast," and there is no doubt that the actinomyces group is closely related through intermediate forms with the tubercle bacillus and the other acid-fast bacilli (*vide* p. 329).

Naked-Eye Characters of the Parasites.—The actinomyces

[1] Madura disease (*vide* p. 377) would, however, conform to this definition, though its etiology, geographical distribution, and pathological features separate it from actinomycosis.

grows in the tissues in the form of little round masses or colonies, which, when fully developed, are easily visible to the naked eye, the largest being about the size of a small pin's head, whilst all sizes below this may be found. When suppuration is present, the colonies lie free in the pus; when there is no suppuration, they are embedded in the granulation tissue, but are usually surrounded by a zone of softer tissue. They may be transparent or jelly-like, or they may be opaque and of various colours—white, yellow, or greenish. The appearance depends upon their age and also upon their structure, the younger colonies being more or less transparent, the older ones being generally opaque. They are generally of soft, sometimes tallow-like, consistence, though sometimes in the ox they are gritty, owing to the presence of calcareous deposit. They may be found in the pus by spreading it out in a thin layer on a glass slide and holding it up to the light (Fig. 89). They are sometimes described as being of a distinctly yellow colour, but this is only occasionally the case; in fact, in the human subject they occur much more frequently as small specks of semi-translucent appearance, and of greenish-grey tint.

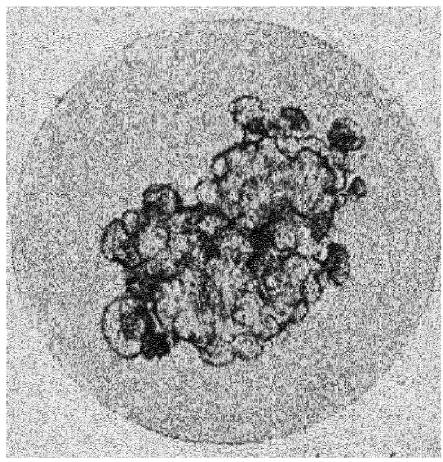


FIG. 89.—Low-power view of untreated colony of actinomyces in pus. ×20.

Microscopical Characters.—Three different morphological elements have been recognised, namely, filaments, spores or conidia, and clubs.

1. The *filaments* are comparatively thin, measuring about 0·6 to 1 μ in diameter, but they are often of considerable length. They are composed of a central protoplasm enclosed by a sheath. The latter, which is most easily made out in the older filaments with granular protoplasm, occasionally contains granules of pigment. In the centre of a colony developing in the tissues the filaments interlace with one another, and form an irregular network which may be loose or dense; at the periphery they are often arranged in a somewhat radiating manner, and run outwards in a wavy or even spiral course. They also show true branching, a character which at once distinguishes them from the ordinary bacteria. Between the filaments there is a finely granular or homogeneous ground

substance. Most of the colonies at an early stage are chiefly constituted by filaments loosely arranged; but later, part of the growth may become so dense that its structure cannot be made out. This dense part, starting excentrically, may grow round the colony to form a hollow sphere, from the outer surface of which filaments radiate for a short distance (Fig. 90). The filaments usually stain uniformly in the younger colonies and are Gram-positive, but often the staining by this method is

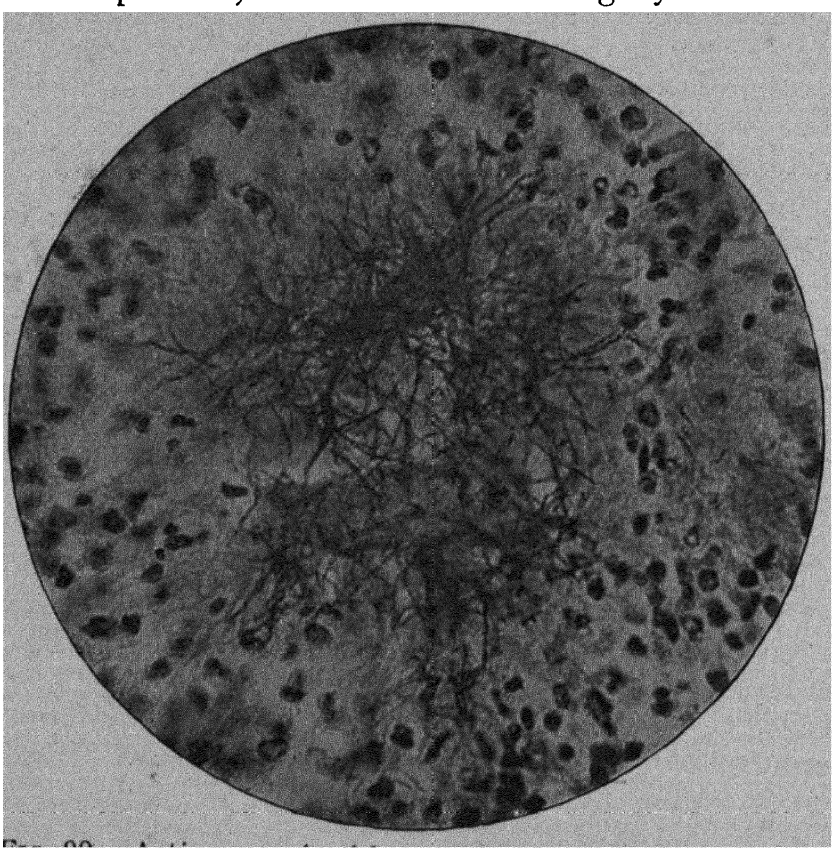


FIG. 90.—Actinomycosis of human liver, showing a colony of the parasite composed of a felted mass of filaments surrounded by pus. Paraffin section; stained by Gram's method and safranin. ×500.

irregular, portions of a filament staining violet, while other parts are decolorised and take up the counter-stain; also a beaded appearance of the filaments may be observed. In the older colonies the filaments may be segmented so as to give the appearance of a chain of bacilli or of cocci, though the sheath enclosing them may generally be distinguished. Rod-shaped and spherical forms may also be seen lying free, some of the latter being conidia.

2. *Spores or Conidia.*—As occurs in other species of streptothrix, some of the filaments of the actinomyces when growing on a culture medium become segmented into rounded spores

or conidia. These structures have been generally regarded as reproductive and capable of forming new colonies on becoming free from the original growth. They have somewhat higher powers of resistance than the filaments, though less than the spores of most of the lower bacteria; cultures containing spores can resist a temperature from five to ten degrees higher than spore-free cultures (Foulerton). An exposure to 75° C. for half an hour is sufficient to kill most streptothrices or their spores.

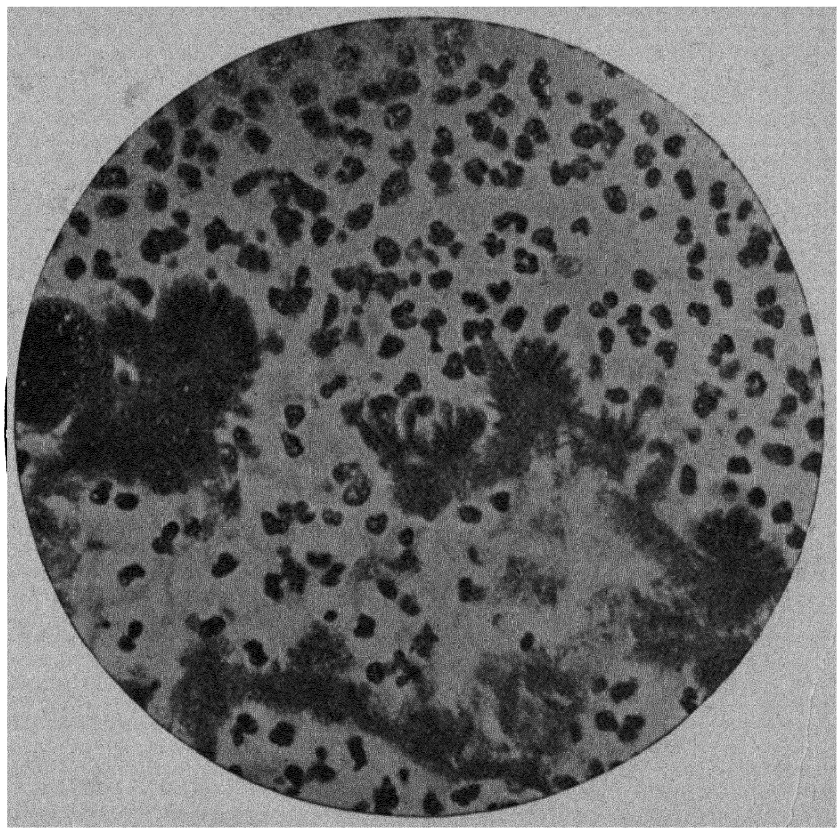


FIG. 91.—Actinomyces in human kidney, showing clubs radially arranged and surrounded by pus. The filaments had practically disappeared.
Paraffin section; stained with hæmatoxylin and rubin. ×500.

The conidia are readily stained by Gram's method. J. H. Wright found, however, in the case of the streptothrix isolated by him from a number of cases (*vide infra*) that there was no distinct evidence of formation of conidia, and it has been questioned whether the common type of actinomyces found in human lesions produces true conidial bodies.

3. *Clubs.*—These are elongated pear-shaped bodies which are seen at the periphery of the colony, and are formed by a sort of hyaline swelling of the sheath around the free extremity of

a filament (Figs. 91, 92). They are usually homogeneous and structureless in appearance. In the human subject the clubs are often comparatively fragile structures, which are easily broken down, and may sometimes be dissolved in water. Sometimes they are well seen when examined in the fresh condition, but in hardened specimens are no longer distinguishable. In specimens stained by Gram's method they are usually not coloured by the violet, but take readily a contrast-stain; sometimes a darkly

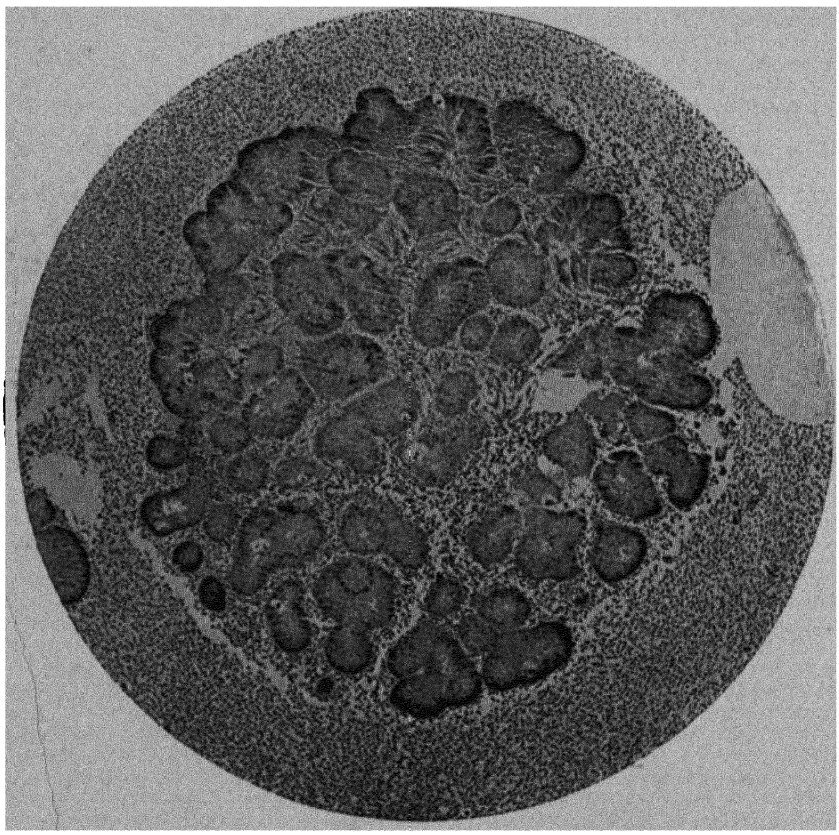


FIG. 92.—Colonies of actinomyces, showing general structural arrangement and clubs at periphery. From pus in human subject. Stained by Gram's method and safranin. ×60.

stained filament can be seen running for a distance in the centre, and may have a knob-like extremity. In many of the colonies in the human subject the clubs are absent. In the ox, on the other hand, where there are much older colonies, the clubs constitute the most prominent feature, and often form a dense fringe around the colony, staining by Gram's method. Occasionally in very chronic lesions in the human subject the clubs stain with Gram's method. Clubs showing intermediate staining reactions have been described in the ox by McFadyean. The club

formation probably represents a means of defence on the part of the parasite against the phagocytes of the tissue. In the majority of cases in the ox no filaments can be detected in the colonies, which are composed almost entirely of radially arranged clubs, and it is from such colonies that Lignières and Spitz have cultivated the *Actinobacillus* (*vide* p. 375).

Tissue Lesions.—In the human subject the lesions are of a chronic inflammatory type, usually ending in a spreading suppuration. In some cases there is a comparatively large production of granulation tissue, with only a little softening in the centre, so that the mass feels solid. In most cases, however, and especially in internal organs, suppuration is the outstanding feature ; this is associated with abundant growth of the parasite in the filamentous form. In an organ such as the liver, multiple foci of suppuration are seen at the spreading margin of the lesion, often presenting a honeycomb appearance, whilst the colonies of the parasite may be seen in the pus with the naked eye. In the older parts the abscesses have become confluent, and formed large areas of suppuration.

In cattle the tissue reaction is more of a formative type, there being abundant growth of granulation tissue, which may result in large tumour-like masses, usually of more or less nodular character, and often consisting of well-developed fibrous tissue containing areas of younger formation, in which, however, irregular abscess formation may be present. The cells immediately around the colonies are usually irregularly rounded, or may even be somewhat columnar in shape, whilst farther out they become spindle-shaped and concentrically arranged. It is not uncommon to find leucocytes or granulation tissue invading the substance of the colonies, and portions of the parasite may be contained within leucocytes or within small giant-cells, which are sometimes present. A similar invasion of old colonies by leucocytes is sometimes seen in human actinomycosis. The disease usually remains quite local, or spreads by continuity. It may produce tumour-like masses in the region of the jaw or neck, or it may specially affect the palate or tongue, in the latter producing enlargement and induration, with nodular thickening on the surface—the condition known as " woody tongue."

Origin and Distribution of Lesions.—The lesions in the human subject may occur in almost any part of the body, the paths of entrance being very various. In many cases the entrance takes place in the mouth or throat—sometimes in the region of a decayed tooth, by the crypts of the tonsil, or by some abrasion of the mucous membrane. Swelling and suppuration may then follow in the vicinity and may spread in various directions, the bones often becoming affected. In a

considerable number of cases the primary lesion is in some part of the intestine, generally the large intestine, and not infrequently in connection with the appendix. A peculiar affection of the intestine has been described, in which slightly raised plaques are found both in the large and small intestines, these plaques being composed almost exclusively of masses of the actinomyces along with epithelial cells. This, however, is a rare condition. The path of entrance may also be by the respiratory passages, the primary lesion being pulmonary or peri-bronchial; extensive suppuration in the lungs may result. Infection may occur by the skin surface, and lastly, by the female genital tract, as in a case recorded by Grainger Stewart and Muir, in which both ovaries and both Fallopian tubes were affected.

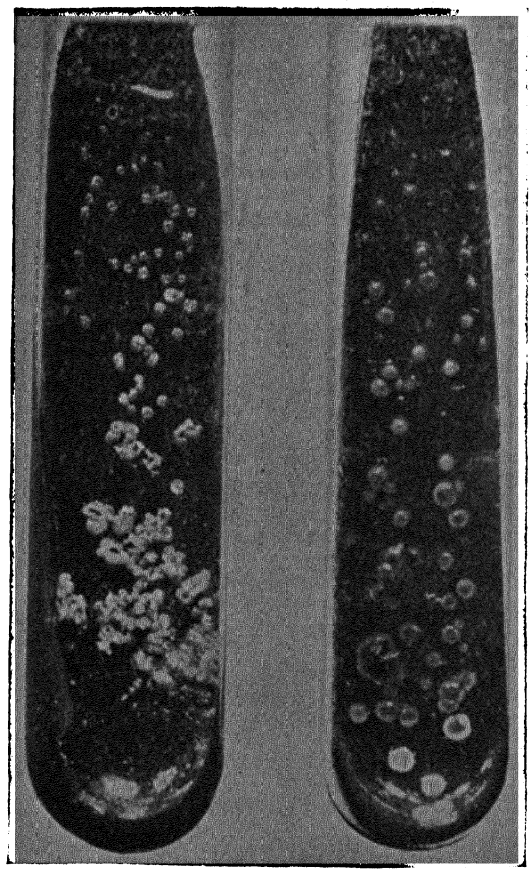
When the parasite has invaded the tissues by any of these channels, secondary or "metastatic" abscesses may occur in internal organs. The liver is the organ most frequently affected, though abscesses may occur in the lungs, brain (where a primary meningitis may also occur), kidneys, etc. In such cases the spread takes place by the blood stream, and it is possible that leucocytes may be the carriers of the infection, as it is not uncommon to find leucocytes in the neighbourhood of a colony containing small portions of the filaments in their interior.

Source of the Parasite.—At one time the view was held that the source of this organism was grain, and especially barley, on which it grew as a saprophyte. Both in the ox and in the pig the parasite has been found growing around fragments of grain, embedded in a mucous membrane. There are besides recorded in the human subject a certain number of cases in which there was a history of penetration of a mucous surface by a portion of grain, and in certain cases also the patient has been exposed to infection from this source. It has been shown, however, that the type of streptothrix isolated from the majority of human cases is a strict parasite, incapable of growing at a temperature much below that of the body and therefore unlikely to exist under saprophytic conditions. A certain amount of evidence has been collected to show that this organism may possibly occur as an inhabitant of the alimentary canal. It has been shown, for example, by Lord, that mycelial organisms similar to actinomyces may be found in carious teeth and in the tonsillar crypts, and he has produced in animals inoculated intraperitoneally with these organisms, granulomatous lesions containing typical actinomyces colonies made up of mycelium and peripheral clubs. Tunnicliff has also described an actinomyces-like organism in tonsillar "granules." Penetration of a mucous

surface by a fragment of grain may act merely by facilitating the invasion of the organism and its establishment in the tissue.

Cultivation (for methods of isolation see later).—The descriptions of the cultures obtained by various investigators differ in essential particulars, and there is no doubt that the organisms described are different. The following classical types may be described:

Streptothrix actinomyces (*Boström*). — On *agar* or *glycerin agar* at 37° C., growth is generally visible on the third or fourth day in the form of little transparent drops which gradually enlarge and form rounded projections of a reddish-yellow tint and somewhat transparent appearance, like drops of amber. The growths tend to remain separate, and even when they become confluent, the nodular character is maintained. They have a tough consistence, being with difficulty broken up, and adhere firmly to the surface of the agar. Older growths often show on the surface a sort of corrugated aspect, and may sometimes present the appearance of having been dusted with a brownish-yellow powder (Fig. 93).



A B

FIG. 93.—Cultures of Streptothrix actinomyces (Boström) on glycerin agar, of about three weeks' growth. The growth in A is at places somewhat corrugated on the surface. Natural size.

In the cultures at an early stage the growth is composed of branching filaments, which stain uniformly (Fig. 94), but later some of the filaments may show segmentation into bacillary and coccal structures, and superficial filaments may show conidia formation. Slight bulbous thickenings may be seen at the end of some of the filaments, but true clubs have not been observed.

In *gelatin* the same tendency to grow in little spherical masses

is seen, and the medium becomes very slowly liquefied. When this occurs the liquefied portion has a brownish colour and somewhat syrupy consistence, and the growths may be seen at the bottom, as little balls, from the surface of which filaments radiate.

Inoculation experiments have, on the whole, given negative results, and it has become doubtful whether this organism really plays a causal rôle in actinomycosis.

Streptothrix actinomyces (Israel and Wolff).—The organism obtained in culture by Israel and Wolff (*vide infra*) is probably the same as the one which was later described in detail by J. H. Wright, who obtained it in pure condition from fifteen different cases of the disease. It differs markedly from Boström's organism in being almost an obligate anaerobe and in ceasing to grow at a temperature a little below that of the body. Under ordinary aerobic conditions either no growth occurs or it is of a very slight character. On the surface of agar under anaerobic conditions the organism produces dense rounded colonies of greyish-white colour, which sometimes assume a rosette form. A special feature of the growth was described by Wright, namely, that in a shake culture in glucose agar the colonies are most numerous and form a dense zone about half an inch from the surface of the medium, that is, at a level where there is presumably a mere trace of oxygen obtainable (Fig. 95). The organism is apparently micro-aerophilic and not a complete anaerobe. In bouillon, growth takes place at the bottom of the medium in rounded masses which afterwards undergo disintegration. Wright found that, when the organism was grown in the presence of serum or other animal fluids, the formation of true clubs occurred at the extremity of some of the filaments (Fig. 96).

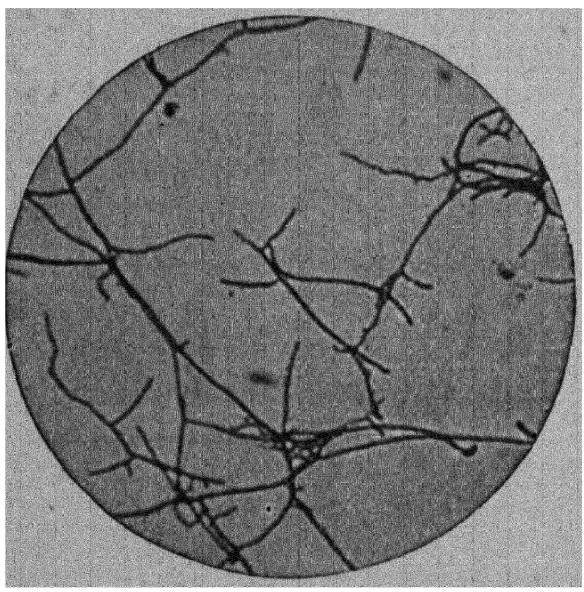


FIG. 94.—Actinomyces, from a culture on glycerin agar, showing the branching of the filaments. See also Plate III., Fig. 10. Stained with fuchsin. ×1000.

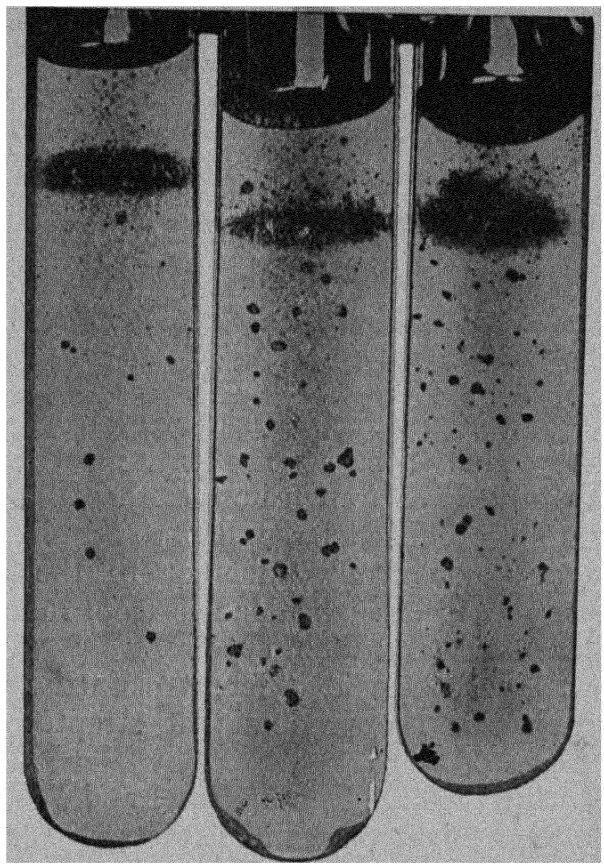


FIG. 95.[1]—Shake cultures of actinomyces in glucose agar, showing the maximum growth at some distance from the surface of the medium.

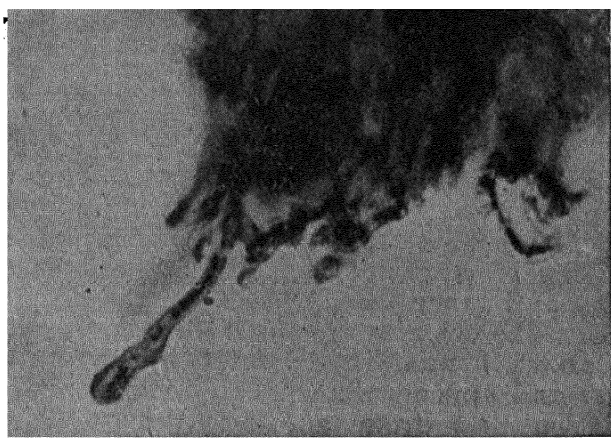


FIG. 96.—Section of a colony of actinomyces from a culture in blood serum, showing the formation of clubs at the periphery. ×1500.

[1] For Figs. 95 and 96 we are indebted to Dr. J. Homer Wright of Boston, U.S.A.

The views of Wright have been supported by the observations of Harbitz and Gröndahl on actinomycosis in Norway. They obtained pure cultures from ten different cases, and in each instance the organism grew only under anaerobic conditions and presented the characters described above. They also obtained the same organism in culture from the disease in the ox. Henry cultivated from actinomycotic meningitis an organism which is a strict anaerobe and which exhibits similar characters.

More recently, Colebrook in this country has studied twenty-four strains of human origin. Of these, twenty-one conformed in their general characters and resembled the Israel and Wolff type. Of the remaining three strains, two showed slight differences from this type in the characters of their growths on medium. The third strain differed entirely from the others in being aerobic. On glucose agar it produced large, flat, rosette-like colonies of tough consistence and firmly adherent to the medium. A series of five strains isolated by Mackie from human cases all conformed to the Israel and Wolff type.

Observations by workers in different countries demonstrate that this organism is the predominant streptothrix type in human infections.

Inoculation with the organism of Israel and Wolff in various animals, including guinea-pigs and rabbits, has given rise to granulomatous nodules, in which the characteristic colonies are present, though the lesions usually have not a progressive character.

Bacillus actinomycetum comitans.—It is of particular biological interest that associated with the mycelial organism in actinomycotic lesions there frequently occurs a minute non-motile Gram-negative coccoid or cocco-bacillary organism measuring often not more than 0·5 μ in its longest diameter and resembling in morphology the Bacillus melitensis (*q.v.*). This concomitant organism was first described in detail by Klinger, who named it *B. actinomycetum comitans*. Colebrook has observed it in the majority of the cases studied by him, and it has also been noted by Mackie. This organism can be cultivated separately from the actinomyces, both aerobically and anaerobically, and growth occurs on ordinary media at 37° C. The colonies are small, discrete, semi-transparent whitish discs about 1 mm. in diameter when fully grown, and contrast in size and appearance with the colonies of the mycelial organism. In cultures from actinomycotic lesions the small colonies of the concomitant may often be seen in groups round the larger raised colonies of the actinomyces. It is comparatively easy to separate this organism from the actinomyces by the usual methods of obtaining pure cultures, especially as it grows aerobically in contrast with the anaerobic growth of the common types of actinomyces, but it may be difficult to obtain pure growths of

the mycelial organism free from the concomitant. In the case of two strains of actinomyces studied by Mackie, it was found impossible to separate the mycelial organism from the concomitant, even after repeated plating and sub-culturing single isolated colonies. A growth of the latter invariably developed from the mycelial organism. One of these strains, however, when maintained in culture for two years was found to be ultimately free from the concomitant. It has been suggested that it may be a derivative or variant of the mycelial organism and not an independent species. On the other hand, its biological characters are so entirely different from the actinomyces that it seems justifiable to regard it as a separate organism. It cannot be identified with the actinobacillus (*vide infra*). Inoculation of animals with pure cultures of this concomitant organism produces no specific effects. It is impossible at present to offer any satisfactory explanation of its occurrence.

Actinobacillus (Lignières and Spitz).—This organism was cultivated by these observers from a number of cases of actinomycosis in the ox, in which no filaments could be detected in the granules. It grows readily on most ordinary media. It is a small bacillus, measuring about 1·5 μ in length and 0·4 μ in thickness, Gram-negative and non-motile. On agar it forms in the primary cultures rounded semi-transparent colonies which reach 1·5 mm. in diameter; in subcultures it forms a continuous layer of similar character. Subcutaneous injection in the sheep and ox, and intraperitoneal injection in the guinea-pig, give rise to lesions in which the characteristic granules with clubs are reproduced. These results have been substantially confirmed by F. Griffith, who obtained a similar organism in twenty-three out of forty cases of bovine actinomycosis. According to this observer, the majority of cases of bovine "actinomycosis" in this country are due to the actinobacillus. The term "actinobacillosis" might be more applicable to this condition.

Other Pathogenic Streptothrices.—Gasperini has described several varieties of *Actinomyces bovis* according to the colour of the growths, and reference has been made above to the varieties found in human actinomycosis. Furthermore, a considerable number of streptothrices has been found in cases of disease in the human subject, the associated lesions varying in character from tubercle-like nodules on the one hand to suppurative processes on the other The organisms cultivated from such sources differ according to their microscopic characters (for example, some form "clubs" whilst others do not), according to their conditions of growth, staining reactions, etc. Of these only a few examples need here be mentioned, but it may be noted that the importance of the streptothrices as causes of disease is constantly being extended. A species of streptothrix was cultivated by Eppinger from a brain abscess, and called by him

"Cladothrix asteroides," from the appearance of its colonies on culture media. A case of general streptothrix infection in the human subject described by Stuart M'Donald was probably due to the same organism as Eppinger's. In the tissues it grows in a somewhat diffuse manner, and does not form clubs; in rabbits and guinea-pigs it produces tubercle-like lesions. Flexner observed a streptothrix in the lungs associated with lesions somewhat like a rapid phthisis, and applied the name "pseudo-tuberculosis hominis streptothricea"; an apparently similar condition has been described by Buchholz. Berestnew cultivated two species of streptothrix from suppurative lesions, one of which is acid-fast and grows only in anaerobic conditions. Birt and Leishman described another acid-fast streptothrix obtained from cirrhotic nodules in the lungs of a man. This organism grows readily on ordinary media, forming a white powdery growth which afterwards assumes a pinkish colour; it is pathogenic for guinea-pigs, in which it causes caseous lesions. There is, further, the streptothrix maduræ described below.

In diseases of the lower animals several other forms have been found. For example, a streptothrix has been shown by Nocard to be the cause of a disease of the ox—"farcin du bœuf"—a disease in which also there occur tumour-like masses of granulation tissue. Dean has cultivated from a nodule in a horse another streptothrix, which produces tubercle-like nodules in the rabbit with club-formation; it has close resemblances to the organism of Israel and Wolff. The so-called diphtheria of calves and "bacillary necrosis" in the ox are probably both produced by a streptothrix or leptothrix, which grows diffusely in the tissues in the form of fine felted filaments. Further investigation may show that some of these or other species may occur in the human subject in conditions which are not yet differentiated.

Methods of Examination and Diagnosis.—As actinomycosis cannot be diagnosed with certainty apart from the discovery of the parasite, a careful examination of the pus in obscure cases of suppuration should always be undertaken. As already stated, the colonies may be recognised with the naked eye, especially when some of the pus is spread out on glass. If some of these are washed in salt solution and examined unstained, the clubs, if present, are at once seen on microscopic examination; several examinations, however, may be necessary. To study the filaments, a colony should be broken down by crushing it between two slides or under a cover-slip on a slide, dried, and stained by Gram's method. While films of pus made at random in the usual way and suitably stained may in certain cases reveal the organism, it is essential that actual granules or colonies should be examined. In the case of sections of the tissues, Gram's method with a contrast-stain yields excellent staining of the mycelium. In the ox the clubs are strikingly demonstrated by staining with carbol-fuchsin and then decolorising with picric alcohol; or the preparation may be decolorised with 1 per cent. sulphuric acid and then contrast-stained with methylene-blue.

Cultures should be made both under aerobic and anaerobic conditions on glucose- or glycerin-agar plates. For this purpose the medium should be inoculated directly from the colonies, and it is advisable to transfer whole colonies from the pus to the plates. Owing to the slow growth of the actinomyces, however, the obtaining of pure cultures is somewhat difficult, unless the pus is free from

contamination with other organisms. When other organisms are present the granules should be picked out with a loop and added to sterile saline in a tube, then washed with several changes of saline. They are then shaken in absolute alcohol for half a minute, washed again in three changes of saline, and finally transferred to the culture medium. M. Gordon has recommended for primary cultures from pus, the use of blood bouillon (in tubes) under a layer of sterile paraffin to secure anaerobiosis if necessary. In this medium the organism grows well in the form of granules. If a mixed growth results with other organisms present, the colonies in the medium, after washing with sterile saline, are transferred to plates, and in this way a pure growth can be obtained.

Leptothrix.—The biological relationships of this type of organism are dealt with in Chapter I. (p. 16). The designation, leptothrix, has been applied to those filamentous organisms which generally resemble the streptothrices but differ from the latter in the absence of branching. A leptothrix type is a common inhabitant of the mouth and has been spoken of as L. buccalis ; it may be found in the tartar deposits on the teeth This organism seems to flourish, especially in the pockets between the gums and teeth in pyorrhœa alveolaris and its growth has been regarded as a factor in tartar formation which is so marked in pyorrhœa (Bulleid). A leptothrix may also occur in the tonsillar crypts. Concretions in the lachrymal duct have been found to be largely composed of growths of this type of organism (Mackie). Leptothrices have also been recorded in inflammatory and suppurative conditions in the region of the mouth and throat, but the pathogenicity of these organisms seems to be relatively weak. Gifford has described a leptothrix in a case of recurrent conjunctivitis. The leptothrix group has not been studied in sufficient detail from the biological standpoint to merit any classification. The organisms are usually observed in the form of elongated unbranched filaments which are typically Gram-positive. Shorter forms may also be noted similar to large bacilli. They are usually non-motile. Both aerobic and anaerobic strains have been described. Cultures on gelatin may produce liquefaction. Pigment-producing types have been noted. Free spores have also been observed (Gifford).

Madura Disease

Madura disease or mycetoma resembles actinomycosis both as regards the general characters of the lesions and the occurrence of the parasite in the form of colonies or "granules." There is no doubt, however, that the two conditions are distinct, and it is also established that the two varieties of Madura disease (*vide infra*) are produced by different organisms. This disease is comparatively common in India and in various other parts of the tropics : it has also been met with in Algiers and in America. Its course is of an extremely chronic nature, and though the local disease is incurable except by operation, the parasite never produces secondary lesions in internal organs.

Vincent also found that iodide of potassium, which has a high value as a therapeutic agent in many cases of actinomycosis, had no effect in the cases of Madura disease studied by him. It most frequently affects the foot; hence the disease is often spoken of as "Madura foot." The hand is rarely affected. In the parts affected there is a slow growth of granulation tissue which has an irregularly nodular character, and in the centre of the nodules there occurs purulent softening, which is often followed by the formation of fistulous openings and ulcers. There are great enlargement and distortion of the part and frequently caries and necrosis of the bones. Within the softened cavities and also in the spaces between the fibrous tissue, small rounded bodies or granules, bearing a certain resemblance to the actinomyces, are present. These may have a yellowish or pinkish colour, compared from their appearance to fish roe, or they may be black like grains of gunpowder, and may by their conglomeration form nodules of considerable size. Hence a *pale variety* and a *black variety* of the disease have been distinguished; in both varieties the granules mentioned reach a rather larger size than in actinomycosis. These two varieties will be considered separately.

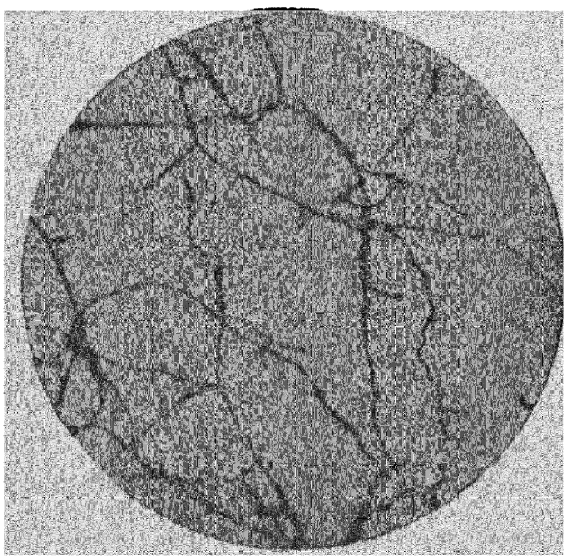


FIG. 97.—Streptothrix maduræ, showing branching filaments. From a culture on agar.
Stained with carbol-thionin-blue. ×1000.

Pale Variety.—When the roe-like granules are examined microscopically they are found, like the actinomyces, to show in their interior an abundant mass of branching filaments with mycelial arrangement. There may also be present at the periphery club-like structures as in actinomycosis; sometimes they are absent. These structures often have an elongated wedge-shape, forming an outer zone to the colony, and in some cases the filaments can be found to be connected with them. The colonies are often markedly degenerate, the mycelium may be densely matted and present an almost structureless appearance

in the tissue. Vincent obtained cultures of the parasite from a case in Algiers, and found it to be a distinct species. It is now known as the *Streptothrix maduræ* (*Actinomyces maduræ*). Since then it has been cultivated by various workers. Morphologically it closely resembles the actinomyces, but it presents certain differences in cultural characters. On all the media, growth only takes place in aerobic conditions. On agar the growth somewhat resembles that of Boström's actinomyces and consists of large, raised, knob-like colonies firmly adherent to the medium. The growth, however, develops a pink colour, which is apparently characteristic of the strains that have been isolated. In gelatin there is no liquefaction of the medium. Experimental inoculation of various animals has failed to reproduce the disease.

Black Variety.—The observations of J. H. Wright, who obtained pure cultures of a hyphomycete, show that this variety is a distinct affection from the pale variety. The pigment may be dissolved by soaking the granules for a few minutes in hypochlorite of sodium solution, and the granules may then be crushed out beneath a cover-glass and examined microscopically. The granules are composed of a somewhat homogeneous ground-substance impregnated with pigment, and in this there is a mycelium of thick filaments or hyphæ, many of the segments of which are swollen; at the periphery the hyphæ form a zone with radiate arrangement. In many of the older granules the parasite is largely degenerated and presents an amorphous appearance. Wright planted over sixty of the black granules in various culture media, and obtained cultures of a hyphomycete from about a third of these. The organism grows well on agar, bouillon, potato, etc.; on agar it forms a felted mass of greyish colour, and in old cultures black granules appear amongst the mycelium. Microscopically the parasite appears as a mycelium of thick branching filaments with delicate transverse septa; in the older threads the segments become swollen, so that strings of oval-shaped bodies result. No signs of spore-formation were noted. Inoculation of animals with cultures gave negative results, as did also direct inoculation with the black granules from the tissues. This parasite has been designated *Madurella mycetomi*. Brumpt has distinguished several varieties of parasite concerned in Madura disease, and finds that a pale variety may be produced by a hyphomycete as well as by Vincent's streptothrix; in fact, with the exception of Vincent's organism, all the parasites have been considered by him to be closely allied to aspergillus.

CHAPTER XIV

ANTHRAX

Introductory.—Anthrax is a disease occurring epizootically among the herbivora, especially sheep and oxen, in which animals it has the characters of a rapidly fatal form of septicæmia with splenic enlargement, attended by an extensive multiplication of the bacilli in the blood. The disease does not occur as a natural infection from man to man, but may be communicated to him directly or indirectly from animals, and it may then appear in one of three forms. In the human subject the bacilli are much more restricted to the local lesions than is the case in the ox.

Historical Summary.—Historical researches leave little doubt that from the earliest times anthrax has occurred among cattle. For a long time its pathology was not understood, and it went by many names. Pollender in 1849 pointed out that the blood of anthrax animals contained numerous rod-shaped bodies which he conjectured had some causal connection with the disease. In 1863 Davaine announced that they were bacteria, and applied the name *Bacillus anthracis*. He stated that unless blood used in inoculation experiments on animals contained them death did not ensue. Though this conclusion was disputed, still by the work of Davaine and others the causal relationship of the bacilli to the disease had been nearly established when the work of Koch appeared in 1876. This not only did much to clear up the whole subject, but formed the starting-point of the science of bacteriology. Koch confirmed Davaine's view that the bodies were bacteria. He observed in the blood of anthrax animals the appearance of division, and from this deduced that multiplication took place in the tissues. He observed them under the microscope dividing outside the body, and noticed spore-formation taking place. He also isolated the bacilli in pure culture outside the body, and, by inoculating animals with them, produced the disease artificially. Koch's observations were, shortly afterwards, confirmed in the main by Pasteur, though controversy arose between them on certain minor points. Moreover, further research showed that the disease could be produced in animals by feeding them with spores, and thus the way in which the disease might spread naturally was explained.

Bacillus Anthracis.—Anthrax as a disease in man is of comparative rarity. Not only, however, is the bacillus anthracis

easy of growth and recognition, but in its growth it illustrates many of the general morphological characters of the whole group of bacteria, and it has therefore been of great use to the student. Further, its behaviour when inoculated in animals illustrates many of the points raised in connection with the general pathogenic effects of bacteria. Hence an enormous amount of work has been done in investigating it in all its aspects.

If a drop of blood is taken immediately after death from an auricular vein of an ox, for example, which has died from anthrax, it will be found to contain a great number of large non-motile bacilli. On staining with watery methylene-blue, the characters of the bacilli can be better made out. They are about 1·2 μ thick, and 6 to 8 μ long, though both shorter and longer forms also occur. The ends are sharply cut across, or may be slightly dimpled so as to resemble somewhat the proximal end of a phalanx. Their protoplasm is very finely granular, and very frequently appears surrounded by a capsule, whose external margin is often not, however, so well defined as in the case of the pneumococcus. When several bacilli lie end to end in a chain, the capsule seems common to the whole chain. They stain well with all the basic aniline dyes and are Gram-positive. To demonstrate the capsule the preparation is well stained with aniline-oil gentian-violet solution, rapidly differentiated in water acidulated with acetic acid, and mounted in water.

Methylene-blue Reaction.—This was introduced independently by McFadyean and by Heim with a view to the easy recognition of the bacilli in blood or other body fluids, and depends on a disintegration of the bacillary capsules which occurs when these are imperfectly fixed. Imperfect fixation is attained by drying a blood film on a *slide* and holding it three times for a second in a flame, film upwards (too great heating fixes the capsules and prevents the reaction from occurring). The preparation is stained for a few seconds with an old solution of methylene-blue, 1 per cent. in water (*i.e.*, with a methylene-blue possessing polychromatic qualities, see p. 105). It is washed in water and dried with filter paper—a cover-glass is not applied. In such a preparation, between and near the bacteria there is a varying amount of an irregularly disposed amorphous or finely granular material of a violet or reddish-purple tint. Frequently the colour reaction in the preparation is so marked as to be recognisable by the naked eye. McFadyean states that this reaction does not occur with putrefactive or other bacteria which might be present under circumstances where the recognition of the anthrax bacilli is the question under consideration. Further reference is made to capsulation later.

Plate Cultures.—From a source such as that indicated, it is

easy to isolate the bacilli by making agar plate-cultures. If, after twelve hours at 37° C., these be examined under a low objective, colonies will be observed. They are to be recognised by beautiful wavy wreaths, like locks of hair, radiating from the centre and apparently terminating in a point which, however, on examination with a higher power, is observed to be a filament which turns upon itself (Fig. 98). Graham-Smith (*vide* p. 5) attributes the appearance to the toughness of the bacterial envelope, which prevents the separation of individuals from one another after division. Thus the colony consists of a continuous convoluted chain of bacilli. The colonies are suitable for making impression preparations which preserve permanently the appearances described. On examining such with a high power, the wreaths are seen to be made up of bundles of long filaments lying parallel with one another, each filament consisting of a chain of bacilli lying end to end, and similar to those observed in the blood (Fig. 99).

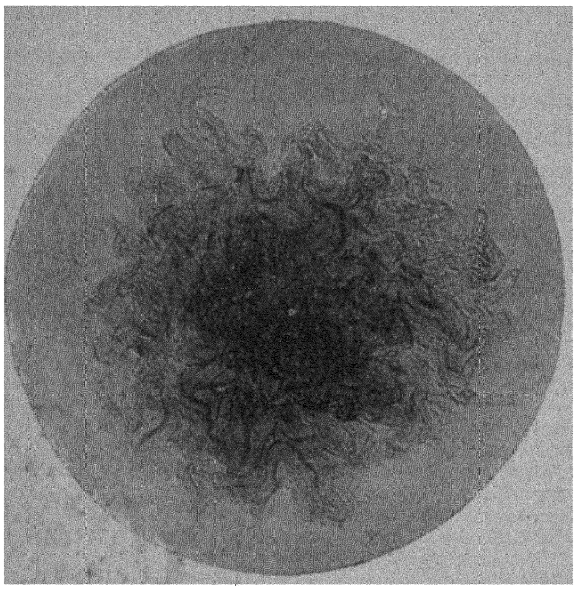


Fig. 98.—Surface colony of the anthrax bacillus on an agar plate, showing the characteristic appearance. ×30.

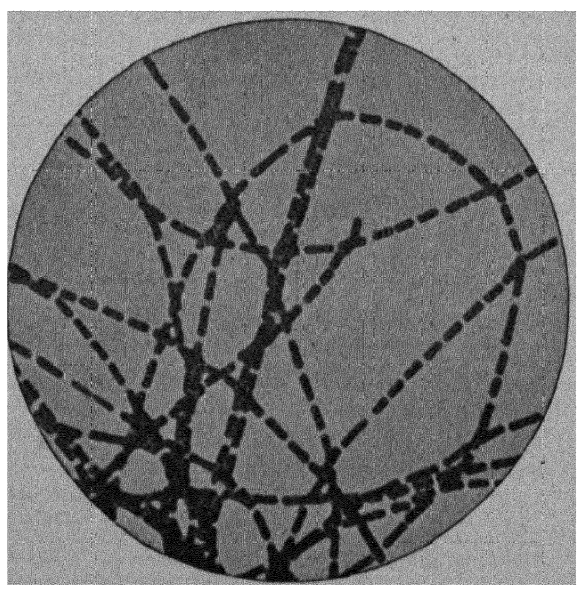


Fig. 99.—Anthrax bacilli arranged in chains, from a twenty-four hours' culture on agar at 37° C.
Stained with fuchsin. ×1000.

To make an impression-preparation a cover-glass is carefully cleaned and flamed; it is then placed on the surface of the medium and gently pressed down on the colony. The edge is then raised by a

sterile needle, it is held with forceps, dried high over the flame, and treated as an ordinary cover-glass preparation.

On gelatin plates, after from twenty-four to thirty-six hours at 20° C., the same appearances manifest themselves, and later they are accompanied by liquefaction of the gelatin. In gelatin plates, however, instead of the characteristically wreathed appearance at the margin, the colonies sometimes give off radiating spikelets irregularly jointed, nodulated, and whorled, which produce a star-like form. These spikelets are composed of spirally twisted threads.

From plates the bacilli can be easily isolated, and the appearances of pure cultures on various media studied.

Agar slope cultures exhibit a thick felted growth, the edges of which show the wreathed appearance seen in plate cultures. On examining the growth by transmitted light it presents a ground-glass appearance. The organism grows readily on *blood serum* and *potato*, but the cultures show no special characteristics.

In *gelatin* stab cultures, the characteristic appearance can be best observed when a low proportion, say, 7½ per cent., of gelatin is present, and when the tube is directly inoculated from anthrax blood. In about two days there radiate out into the medium from the needle track numerous very fine spikelets which enable the cultures to be easily recognised. These spikelets are longest at the upper part of the needle track (Fig. 100). Spread takes place on the surface of the gelatin, and here liquefaction commences, and gradually extends down the stab and out into the medium, till the whole of the gelatin may be liquefied.

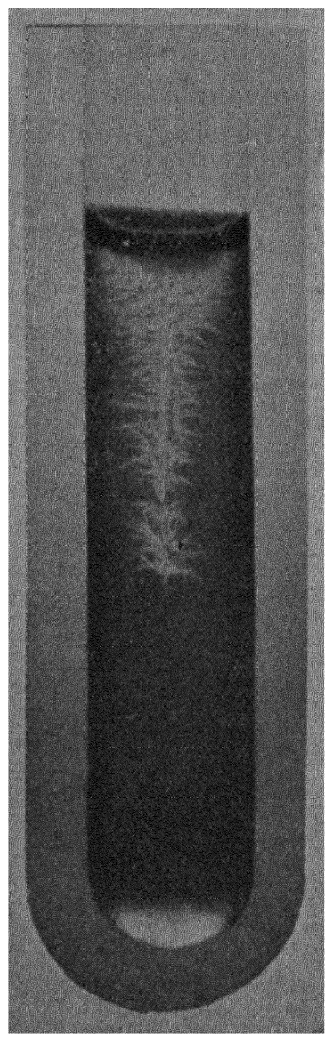


FIG. 100.—Stab culture of the anthrax bacillus in peptone-gelatin; seven days' growth. It shows the "spiking," and also, at the surface, commencing liquefaction. Natural size.

In *bouillon*, after twenty-four hours' incubation at 37° C.,

there is usually the appearance of irregular spiral threads suspended in the liquid. These, on being examined, are seen to be made up of bundles of parallel chains of bacilli. Later, growth is more abundant, and forms a flocculent mass at the bottom of the fluid.

The Biology of the B. Anthracis.—Koch found that the bacillus anthracis grows best at a temperature of 35° C. Multiplication does not take place below 12° C. nor above 45° C. In the spore-free condition the bacilli have comparatively low powers of resistance. They do not stand long exposure to 60° C., and if kept at ordinary temperature in the dry condition they are usually found to be dead after a few days. The action of the gastric juice is rapidly fatal to them, and they are accordingly destroyed in the stomachs of healthy animals. They are also soon killed in the process of putrefaction. They can, however, be cooled below the freezing-point without dying. The bacillus can grow without oxygen, but some of its vital functions are best carried on in the presence of this gas. Thus in anthrax cultures the liquefaction of gelatin always commences at the surface and spreads downwards. Growth is more rapid in the presence of oxygen, and spore formation does not occur in its absence. The organism may be classed as a facultative anaerobe.

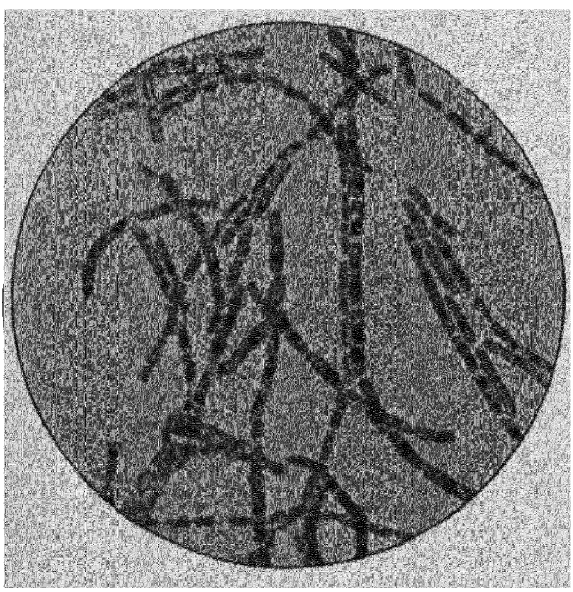


FIG. 101.—Anthrax bacilli containing spores (the darkly coloured bodies); from a three days' culture on agar at 37° C. See also Plate III., Fig. 2.
Stained with carbol-fuchsin and methylene-blue. ×1000.

Sporulation.—Under certain circumstances sporulation occurs in anthrax bacilli. The morphological appearances are of the ordinary kind. A little highly refractile speck appears in the protoplasm about the centre of the bacillus; this gradually increases in size until it forms an oval body of about the same thickness as the bacillus lying in the bacillary protoplasm (Fig. 101). The latter gradually loses its staining capacities and finally disappears. The spore thus lies free as an oval

highly refractile body which does not stain by ordinary methods, but which can be stained by the special methods described for such a purpose (p. 110). When the spore is again about to assume the bacillary form the capsule is apparently absorbed, and the protoplasm within grows out, taking on the ordinary rod-shaped form.

It is generally agreed that sporulation never occurs within the body of an animal suffering from anthrax. Koch attributed this to the absence of free oxygen, which he found necessary to the occurrence of spores in cultures outside the body. Many, however, are inclined to assign as the cause of sporulation the absence of the optimum pabulum. Besides these conditions there is another factor necessary to sporulation, namely, a suitable temperature. The optimum temperature for spore production is 30° C. Koch found that spore-formation did not occur below 18° C. Above 42° C. not only does sporulation cease, but Pasteur found that if bacilli were kept at this temperature for eight days they did not regain the capacity when again grown at a lower temperature. In order to make them again capable of sporing, it was necessary to adopt special measures, such as passages through the bodies of a series of susceptible animals.

Anthrax spores have extremely high powers of resistance. In a dry condition they will remain viable for a year or more. Koch found they resisted boiling for five minutes; and dry heat at 140° C. must be applied for several hours to kill them with certainty. Unlike the bacilli, they can resist the action of the gastric juice for a long period of time. They are often used as test objects by which the action of germicides is judged (see Appendix).

Capsulation.—This is very frequently observed in the B. anthracis both in tissues and in cultures, but the appearances vary under different biological conditions and sometimes capsule formation is absent. The capsule sometimes has as sharp an external contour as occurs in the pneumococcus, but in other cases is not so definitely marked, and sometimes when bacilli are lying together their capsules appear to blend to form a somewhat ill-defined halo. Such variations are associated with slight differences in the naked-eye appearance and physical characters of surface growths. In those where the capsule is indefinite, the growth is moister and more slimy and the edges of the colonies may not present the typical wreathed appearances already described. Such variations have been noted by Preisz as of special frequency in strains deprived of their power of sporulation by heat, and different colonies isolated from such strains may present differences in the character of the capsule. It has been supposed that capacity to produce a well-formed firm

capsule is associated with the possession of special virulence, non-capsulating strains frequently showing low pathogenic qualities. This, however, has been found not to be an invariable rule. According to Ottolenghi, in cultures the capsule is formed from the carbohydrates present.

It is evident from what has been said that modifications in both biological and cultural characters can be artificially originated in anthrax bacilli.

Anthrax in Animals.—Anthrax occurs from time to time epizootically in sheep, cattle, and, more rarely, in horses and deer; it is world-wide in its distribution. All the countries of Europe are from time to time visited by the disease, but in some it is much more common than in others. In Britain the death-rate is small, and often only one animal in a herd is affected, but in France the annual mortality among sheep was formerly about 10 per cent. of the total number in the country, and among cattle 5 per cent. These figures, however, have been largely modified by the system of preventive treatment which will be presently described. In sheep and cattle the disease is specially virulent, and death often occurs with great rapidity. In less acute cases the animal is apparently out of sorts, and does not feed; there is often a sanguineous discharge from the bowels, and bloody mucus may be observed about the mouth and nose. Progressive weakness, with cyanosis, is followed by death in from twelve to forty-eight hours. When the disease is more prolonged, widespread œdema and extensive enlargement of lymphatic glands are marked features; and in the glands, especially about the neck, actual necrosis with ulceration may occur, constituting the so-called anthrax carbuncles. Such subacute conditions are especially found among horses, which are by nature not so susceptible to the disease as cattle and sheep. Occasionally even in susceptible animals recovery takes place.

On post-mortem examination of an ox dead of anthrax, the most noticeable feature—which has given the name "splenic fever" to the disease—is the enlargement of the spleen, which may be two or three times its natural size. It is of dark red colour, and on section the pulp is very soft and friable. A film made from the spleen and stained with watery methylene-blue will be found to contain enormous numbers of bacilli mixed with red corpuscles and leucocytes, chiefly lymphocytes and the large mononucleated variety (Fig. 102). The lymphatic system generally is much affected, especially in less acute cases. The glands, especially the mediastinal, mesenteric, and cervical

glands, are enlarged and surrounded by œdematous tissue, the lymphatic vessels are swollen, and both glands and vessels may contain numberless bacilli. The intestines are enormously congested, the epithelium more or less desquamated, and the lumen filled with a bloody fluid. The changes in other organs are those met with in septicæmia. The blood throughout the body is usually fluid and of dark colour; bacilli can be found

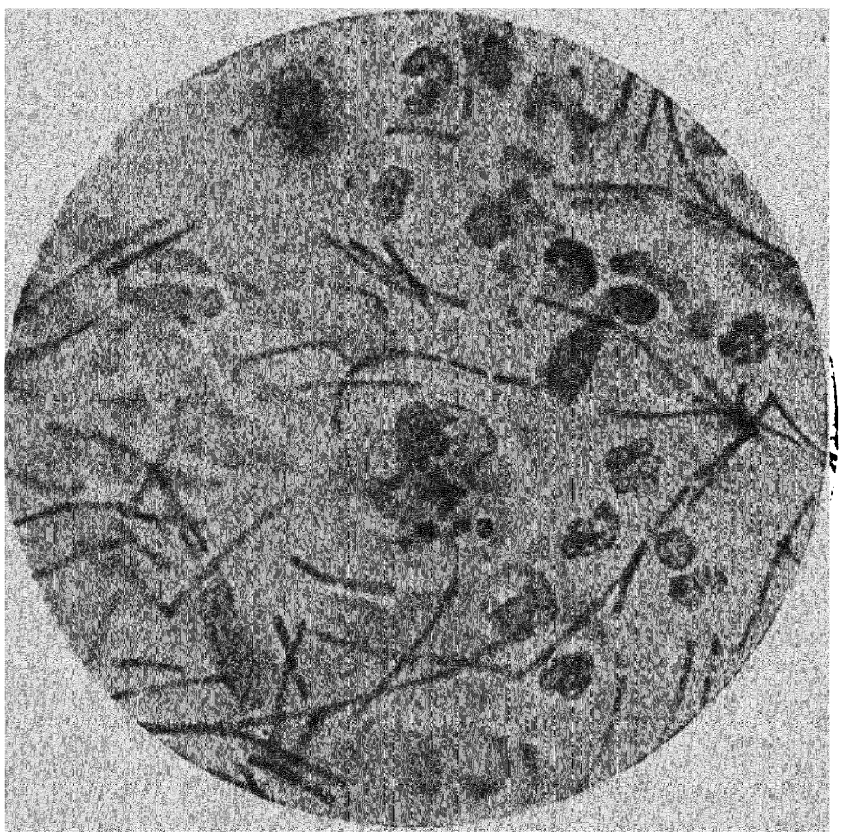


FIG. 102.—Scraping from spleen of guinea-pig dead of anthrax, showing the bacilli mixed with leucocytes, etc. (Same appearance as in the ox.)
"Corrosive-fixed film" stained with carbol-thionin-blue. ×1000.

in it on microscopic examination, but they are specially numerous in the capillaries of internal organs.

Great differences exist in susceptibility to anthrax in different species of animals. Thus the ox, sheep (except those of Algeria, which only succumb to enormous doses of the bacilli), guinea-pig, and mouse are all very susceptible, the rabbit slightly less so. Less susceptible than this group are the horse, deer, and goat, in which the disease occurs from time to time in nature. Anthrax also occurs epizootically in the pig, often from the ingestion of the organs of other animals dead of the disease. It is, however,

doubtful if all cases of disease in the pig described on clinical grounds as anthrax are really such. A careful bacteriological examination is here always advisable, especially of any œdematous infiltration about the throat, or in the neighbouring lymphatic glands; often, in pigs dying of anthrax, bacilli may not occur in the blood. Any hæmorrhagic infarction in the spleen of a suspected animal should be carefully investigated. The human subject may be said to occupy a medium position between the highly susceptible and the relatively immune animals. The white rat is highly immune to the disease, while the brown rat is suscept-

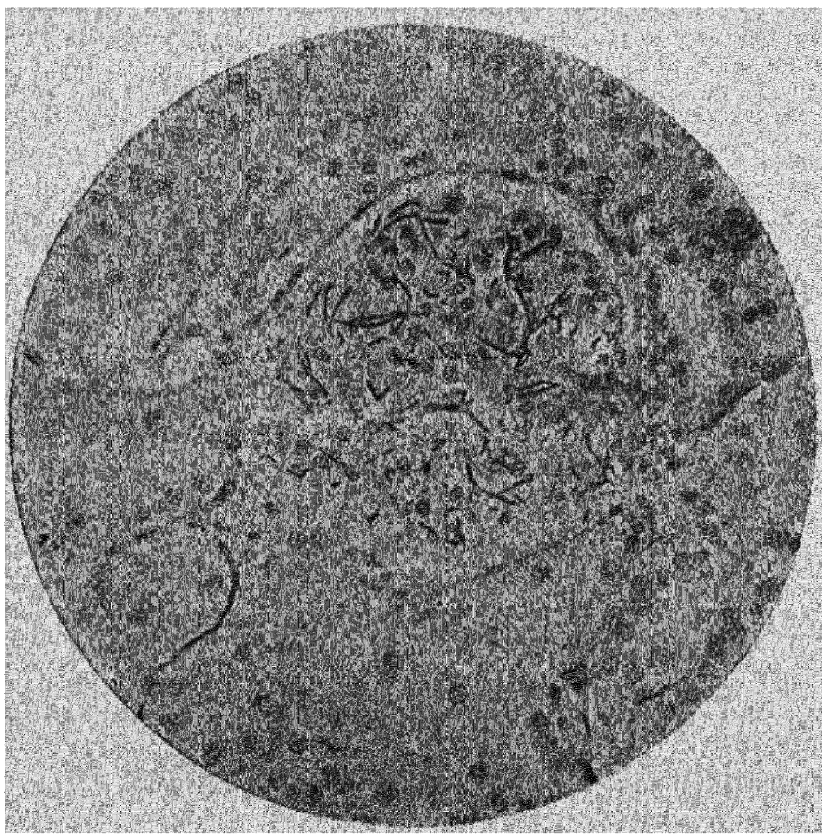


FIG. 103.—Portion of kidney of guinea-pig dead of anthrax, showing the bacilli in the capillaries, especially of the glomerulus. Paraffin section; stained by Gram's method and Bismarck-brown. ×300.

ible. Adult carnivora are also very immune, and birds and amphibia are in the same position.

With these differences in susceptibility there are also great variations in the pathological effects produced in the natural or artificial disease. This is especially the case when we consider the distribution of the bacilli in the bodies of the less susceptible animals. Instead of the widespread occurrence described above, they may be confined to the point where they first gained access

to the body and the lymphatic system in relation to it, or may be only very sparsely scattered in organs such as the spleen (which is often not enlarged), the lungs, or kidneys.

Experimental Inoculation.—Of the animals commonly used in laboratory work, mice and guinea-pigs are the most susceptible to anthrax, and are generally used for test inoculations. If a small quantity of anthrax bacilli be injected into the subcutaneous tissue of a guinea-pig, a fatal result follows, usually within two days. *Post mortem,* around the site of inoculation the tissues, owing to intense inflammatory œdema, are swollen and gelatinous in appearance, small hæmorrhages are often present, and on microscopic examination numerous bacilli are seen. The internal organs show congestion and cloudy swelling, with sometimes small hæmorrhages, and their capillaries contain enormous numbers of bacilli (Fig. 103), as has already been described in the case of the ox ; the spleen also shows a corresponding condition. Highly susceptible animals may be infected by being made to inhale the bacilli or their spores, and also by being fed with spores, a general infection rapidly occurring by both methods. In the case of small animals the results of introducing the bacilli or their spores into the trachea or by feeding have ~~given~~ divergent ~~results~~ in the hands of various observers. Besredka has recently brought forward observations which show that the guinea-pig and the rabbit are susceptible only to inoculation of the skin. When the bacilli are injected intravenously or intraperitoneally, care being taken to avoid contamination of the skin, no harmful result may occur. He holds that an essential condition for infection is the production of a lesion of the skin evidenced by œdema ; thereafter general infection takes place. His results have been confirmed by Balteano, but have been questioned by others.

Anthrax in the Human Subject.—As we have noted, man occupies a middle position in the scale of susceptibility to anthrax. It is always communicated to him from animals directly or indirectly, and usually is seen among those whose trade leads them to handle the carcases or skins of animals which have died of the disease. It occurs in two principal forms, the main difference between which is due to the site of entrance of the organism into the body. In one, the path of entrance is through cuts or abrasions in the skin, or through the hair follicles. A local condition called a " malignant pustule " develops, which may lead to a general infection. This variety occurs chiefly among butchers and those who work among hides (foreign ones especially). In Britain the workers of the latter class chiefly liable are the hide-

porters and hide-workers in South-Eastern London. Occasionally the disease has been contracted from anthrax spores in shaving-brushes made from the bristles of infected animals. In the other variety of the disease the site of infection is the trachea and bronchi, and here a fatal result almost always follows. The cause is the inhalation of dust or threads from wool, hair, or bristles, which have been taken from animals dead of the disease, and which have been contaminated with blood or secretions containing the bacilli, these having afterwards formed spores. This variety is often referred to as "woolsorter's disease," from its occurring in the centres of the woolstapling trade (in England, chiefly in Yorkshire), but it also is found in places where there are hair, brush, or carpet factories.

(1) *Malignant Pustule.*—This usually occurs on the exposed surfaces—the face, hands, forearms, and back, the last being a common site among hide-porters. One to three days after inoculation a small red painful pimple appears, soon becoming a vesicle, which may contain clear or blood-stained fluid; it is rapidly surrounded by an area of intense congestion. Central necrosis occurs, and leads to the malignant pustule proper, which in its typical form appears as a black eschar of irregular shape often surrounded by a ring of vesicles, these in turn being surrounded by a congested area. From this pustule as a centre subcutaneous œdema spreads, especially in the direction of the lymphatics; the neighbouring glands are enlarged. There is usually fever with general malaise. On microscopic section of the typical pustule, the central eschar is noticed to be composed of necrosed tissue and degenerating blood cells; the vesicles are formed by the raising of the stratum corneum from the rete Malpighii. The cells of the latter are swollen and œdematous, the papillæ being enlarged and flattened out and infiltrated with inflammatory exudation which also extends beneath the centre of the pustule. In the tissue next the eschar necrosis is commencing. The subcutaneous tissue is also œdematous, and often infiltrated with leucocytes. The bacilli exist in the periphery of the eschar and in the neighbouring lymphatics, and, to a certain extent, in the vesicles. It is very important to note that widespread œdema of a limb, enlargement of neighbouring glands, and fever may occur while the bacilli are still confined to the immediate neighbourhood of the pustule. Sometimes the pathological process goes no further, the bacilli gradually die out, the eschar becomes a scab, the inflammation subsides, and recovery takes place. In other cases, however, the œdema spreads, invasion of the blood stream may occur, and the patient

dies with, in a modified degree, the pathological changes detailed in regard to the acute disease in cattle. In man the spleen is usually not much enlarged, and the organs generally contain few bacilli.

(2) *Woolsorter's Disease.*—The pathology of this affection was first worked out in this country by Greenfield. The local lesion is usually situated in the lower part of the trachea or in the large bronchi, and is in the form of swollen patches in the mucous membrane, often with hæmorrhage into them—small ulcers may also be seen. The tissues are intensely inflamed, œdematous, and the cellular elements are separated, but there is usually little or no necrosis. There is enormous enlargement and engorgement of the mediastinal and bronchial glands, and hæmorrhagic infiltration of the cellular tissue in the region. There are pleural and pericardial effusions, and hæmorrhagic spots occur beneath the serous membranes. The lungs show great congestion, collapse, and œdema. There may be cutaneous œdema over the chest and neck, with enlargement of glands, and the patient rapidly dies with symptoms of pulmonary embarrassment, and with a varying degree of pyrexia. It is to be noted that in such cases, though numerous bacilli are present in the bronchial lesions, in the lymphatic glands, and affected tissues in the thorax, comparatively few may be present in the various organs, such as the kidney, spleen, etc., and sometimes it may be impossible to find any.

(3) Infection occasionally takes place through the intestine, probably by ingestion of spores as in the case of animals; but this condition is rare. In such cases there occur single or multiple local hæmorrhagic lesions in the intestinal mucous membrane, the central parts of the hæmorrhagic areas tending to be necrotic and yellowish, and there may be a corresponding affection of the mesenteric glands.

A considerable number of cases have been recorded in which hæmorrhagic meningitis, associated with the presence of the anthrax bacilli in large numbers, has occurred as a complication of various primary lesions.

The Spread of the Disease in Nature.—We have seen that the B. anthracis rarely, if ever, forms spores in the body, and if the bacilli could be confined to the blood and tissues of carcases of animals dying of the disease, it is certain that anthrax in an epizootic form would be less frequent. For it has been shown by many observers that in the course of the putrefaction of such a carcase the anthrax bacilli rapidly die out, and that after ten days or a fortnight very few remain. But it must be remem-

bered that while still alive an animal is discharging by the bloody excretions from the mouth, nose, and bowel, myriads of bacilli which may rapidly spore, and thus arrive at a very resistant stage. These lie on the surface of the ground and are washed off by surface water. It is in the condition of spores that they are dangerous to susceptible animals. In the bacillary stage, if swallowed, they will be killed by the acid gastric contents; but as spores they can pass uninjured through the stomach, and gaining an entrance into the intestine, infect its wall, and ultimately reach, and multiply in, the blood. It is known that in the great majority of cases of the disease in sheep and oxen, infection takes place thus from the intestine. In Britain the occurrence of sporadic outbreaks has been attributed to infection by imported feeding-stuffs.

The Disposal of the Carcases of Animals dead of Anthrax.—It is extremely important that anthrax carcases should be disposed of in such a way as to prevent their becoming future sources of infection. If anthrax be suspected as the cause of death, no post-mortem examination should be made, but only a small quantity of blood removed from an auricular vein for bacteriological investigation. If such a carcase be now buried in a deep pit surrounded by quicklime, little danger of infection will be run. The bacilli being confined within the body will not spore, and will die during the process of putrefaction. The danger of sporulation taking place is, of course, much greater when an animal has died of an unknown disease, which, on post-mortem examination, has proved to be anthrax, but similar measures for burial must be here adopted. In some countries anthrax carcases are burned, and this, if practicable, is of course the best means of treating them. The chief source of danger to cattle subsequently, however, proceeds from the infection of fields, yards, and byres with the offal and the discharge from the mouths of anthrax animals. All material suspected of being infected should be burned along with the straw in which the animals have lain. The stalls or buildings in which the anthrax cases have been must be limewashed. Needless to say, the greatest care must be taken in the case of men who handle the animal or its carcase that they have no wounds on their persons, and that they thoroughly disinfect themselves by washing their hands, etc., in 1 : 1000 solution of corrosive sublimate or in liquor cresol. sap. All clothes soiled with blood, etc., from anthrax animals, should be thoroughly boiled or steamed for half an hour before being washed.

Immunity against Anthrax.—Having ascertained that there was ground for believing that in cattle one attack of anthrax protected against a second, Pasteur (in the years 1880–82) elaborated a method by which a mild form of the disease could be given to animals, which rendered harmless a subsequent inoculation with virulent bacilli. He found that the continued

growth of anthrax bacilli at 42° to 43° C. caused them to lose their capacity of producing spores, and also gradually to lose their virulence, so that after twenty-four days they could no longer kill either guinea-pigs, rabbits, or sheep. Such cultures constituted his *premier vaccin,* and protected against the subsequent inoculation with bacilli which had been grown for twelve days at the same temperature, and the attenuation of which had therefore not been carried so far. The latter constituted the *deuxième vaccin.* It was further found that sheep thus twice vaccinated now resisted inoculation with a culture which usually would be fatal. After the efficacy of vaccination in this way had been experimentally established on a large scale, the method was employed as a preventive measure and has since that time been regularly used in France and elsewhere. There is no doubt that the mortality from natural infection has been much diminished by this system. It is to be noted, however, that the state of active immunity passes off—in a considerable proportion of cases at the end of a year.

In France, during the twelve years 1882–93, 3,296,815 sheep were vaccinated, with a mortality, either after the first or second vaccination, or during the subsequent twelve months, of 0·94 per cent., as contrasted with the ordinary mortality in all the flocks of the districts of 10 per cent. During the same time 438,824 cattle were vaccinated, with a mortality of 0·34 per cent., as contrasted with a probable mortality of 5 per cent. if they had been unprotected.

Anti-anthrax sera have been prepared from highly immunised animals, and such sera have been found to possess protective and, to a certain extent, curative properties. In their preparation it is necessary to use living cultures of the bacillus; these are usually employed at first in an attenuated state by Pasteur's method and later in the form of virulent cultures. The method has been combined with the use of an antiserum obtained from an animal already immunised—that is, a combination of active and passive immunisation is carried out. The two best known sera are those of Sclavo and Sobernheim. The former, who found the ass the most suitable animal for immunisation, published favourable results obtained in the treatment of malignant pustule. Out of 164 cases there were only ten deaths, this representing about a fourth of the ordinary mortality in Italy. The serum is administered by intramuscular or by intravenous injection. Sobernheim's serum has been used for protective purposes, the antiserum being injected at the same time as Pasteur's second vaccine. This method has been widely used in Germany and in Brazil, and it is claimed

that it has advantages over Pasteur's, that its application is simpler, one operation instead of two being sufficient, that there is less risk of death following the immunisation procedure, and that the resulting immunity is of higher degree and of more lasting character.

Whilst anti-anthrax sera have markedly preventive properties, it does not seem possible to explain their action by antibodies which can be demonstrated *in vitro*. Sobernheim and others were unable to detect in such sera any trace of special bactericidal action, and Sclavo came to the conclusion that in the action of the serum, substances of the nature of immune-body and complement are not concerned. Opsonic action has been put forward in explanation, but this has not been accepted as sufficient, and the mode of action has not been satisfactorily explained. Anti-anthrax serum may contain a precipitin for the products of the anthrax bacillus (*vide infra*), but this, of course, does not explain the beneficial action of the serum. Other puzzling facts with regard to immunity against anthrax have been established. For instance, the serum of the dog, which has great natural resistance, has little if any bactericidal action, whilst the serum of the susceptible rabbit is capable of killing the organisms.

In connection with the subject of immunity it is to be recognised also that the manner in which the anthrax bacillus produces its pathogenic effects is very imperfectly understood. Toxic action is undoubtedly concerned, as is indicated by the inflammatory œdema which occurs apart from the actual presence of the bacilli, but it has not been found possible to separate toxins from cultures. Filtered bouillon cultures are almost non-toxic, and the dead bacilli themselves likewise have little effect. The facts accordingly suggest that when invading the tissues the bacilli form toxins such as are not produced in cultures. These toxins are considered by Bail to be of the nature of aggressins (p. 165) and in support of this he states that the protective action of an anthrax immune serum is due to its containing anti-aggressins. It may be stated that the supposed aggressins have been obtained by centrifuging œdema fluid or pleural exudate obtained from infective animals, and then killing any remaining bacilli by shaking the fluid with toluol.

Whilst larger susceptible animals can be immunised by the methods above described, it has been found difficult or often impossible to produce immunity by this method in the smaller susceptible animals such as the guinea-pig and the rabbit.

Besredka has found that immunity can be developed when inoculation by the skin is employed, either intracutaneous injection or rubbing a culture over a shaved area of skin, the vaccines as used by Pasteur being successively applied, followed by virulent cultures. Such a method produces an immunity of the skin, and as, according to his view, the other tissues are naturally resistant (*vide supra*) the animal possesses also general immunity. He found also that this immunity of the skin was not attended by the appearance of antibodies in the blood.

Ascoli's Thermo-precipitin Reaction.—This depends on the observation that certain anthrax immune sera produce a precipitin reaction with the products of the B. anthracis. The suspected blood or tissue is boiled for a few minutes in 5 to 10 volumes of normal saline which may be acidulated by one part per thousand of acetic acid ; the fluid is cooled and filtered through paper or asbestos so as to obtain a clear filtrate ; a little of this is then run on to the top of the serum, and a white ring should form immediately at the junction of the fluids. The reaction sometimes occurs with normal sera, but in this case does not appear for a quarter of an hour. It is absolutely necessary that the serum to be used should be previously tested with material derived from an undoubted anthrax case, as only a certain small proportion of immune sera will give the reaction. The reaction seems to depend on an effect produced between the serum and substances derived from the bacilli, as it is most marked with tissues containing numerous organisms. It can be obtained with material which has been kept for six months, and numerous controls made with tissues of animals dying from other diseases are stated to have given negative results.

Methods of Examination.—(*a*) *Microscopic Examination.*—In a case of suspected malignant pustule, film preparations should be made from the fluid in the vesicles or from a scraping of the incised or excised pustule, and stained by Gram's method. In this way practically conclusive evidence may be obtained. McFadyean's methylene-blue method (p. 381) may also be applied. Occasionally bacilli are so scanty that both film preparations made from different parts and even cultures may give negative results, and yet a few bacilli may be found when a section of the pustule is examined. Care ought to be taken in manipulating a pustule, as, otherwise, the diffusion of the bacilli into the surrounding tissues may be aided. The examination of the blood in cases of anthrax in man usually gives negative results, with the exception of very severe cases, when a few bacilli may be found in the blood shortly before death.

(*b*) *Cultivation.*—The material should be stroked on agar plates. After twenty-four hours at 37° C. anthrax colonies will appear, and from their wavy margins can be readily recognised by means of a hand-lens.

While the isolation of the B. anthracis from fresh material is usually easy, great difficulty may be encountered where the organism is to be sought for in a carcase which has been dead for twenty-four hours or longer, as the bacilli rapidly die out or are associated with putrefactive organisms.

(*c*) *Test Inoculation.*—A little of the suspected material mixed with some sterile saline should be injected subcutaneously into a guinea-pig or mouse. If anthrax bacilli are present, the animal usually dies within two days, with the changes in internal organs already described, and the bacilli can be demonstrated in the heart blood. The diagnosis of an organism as the anthrax bacillus cannot be said to be substantiated till its pathogenicity has been proved.

Organisms Biologically allied to the Anthrax Bacillus

It must be recognised that B. anthracis constitutes one species among a group of Gram-positive, aerobic, sporing bacilli which are widely distributed in nature. The anthrax bacillus is the only pathogenic representative of the group. The others are saprophytes, occurring in soil, dust, water, etc. Spores of these organisms are practically ubiquitous and frequently contaminate culture medium, especially when exposed to air. Some of them so closely resemble B. anthracis that, apart from animal inoculation tests, they may be easily mistaken for it. Thus morphological and cultural characters almost identical with those of the anthrax bacillus may be presented by certain of these organisms (*e.g.* the so-called *B. anthracoides*) ; though in some cases motility in culture clearly distinguishes them from B. anthracis. Inoculation of a mouse or guinea-pig, to which B. anthracis is extremely virulent, serves, however, as the essential method of differentiation. It is noteworthy, however, that certain organisms of this type are not entirely devoid of virulence and may on subcutaneous inoculation produce a lethal effect in mice and guinea-pigs, if large doses of cultures are used (Grierson). Even a septicæmic condition may result, as in anthrax, but the organisms in the blood and spleen fail to show McFadyean's reaction. A small dose of a recently isolated anthrax bacillus is, of course, sufficient to cause death in a susceptible animal.

Classical types belonging to this group are B. mycoides, B. subtilis, B. vulgatus. *B. mycoides* is very similar in morphology to B. anthracis, but its colonies on nutrient agar differ from those of the latter in exhibiting a feathery or spiked appearance. *B. subtilis* also resembles the anthrax bacillus in morphology, but the ends are often rounded, there is less tendency to chain formation, and in young cultures the bacilli show active motility due to peritrichous flagella. This organism is strictly aerobic and grows best at low temperatures, *e.g.* about 20° C. A stroke inoculation on culture medium produces an abundant dry, opaque, greyish-white layer of growth without the characteristic wavy or wreathed margin of B. anthracis cultures. A gelatin culture made by stab inoculation consists almost entirely of surface growth owing to the strictly aerobic character of the organism, and there is little or no growth along the needle track. A potato culture consists at first of a moist layer of growth, but later assumes a dry mealy appearance. *B. vulgatus* closely resembles B. subtilis, and is characterised by the marked wrinkling and folding of the growths on artificial media.

CHAPTER XV

TYPHOID FEVER—BACILLI ALLIED TO THE TYPHOID BACILLUS

Introductory.—The organism now known as the Bacillus typhosus was first described in 1880–81 by Eberth, who observed its microscopic appearance in the intestinal ulcers and in the spleen in cases of typhoid fever. It was first isolated (from the spleen) in 1884 by Gaffky, and its cultural characters were then investigated. In 1885 Escherich described the Bacillus coli communis, now the classical type of those organisms designated collectively B. coli, which occur normally in the intestine and to which the typhoid bacillus is biologically related. While ordinarily the B. coli is a harmless saprophyte, it may manifest pathogenic properties under certain conditions. These two bacilli belong to a group of organisms isolated from various intestinal infections, which bear resemblances to one another, and whose differentiation is in certain cases a matter of considerable difficulty. Among other members of this group are the paratyphoid bacilli and the B. enteritidis of Gaertner, the bacillus of hog cholera, and the dysentery bacilli.

The general characters of the *coli-typhoid* group are as follows: the organisms, which are microscopically indistinguishable except in regard to motility (and the possession of flagella), which varies with different members, are small non-sporing bacilli, which in cultures often show variation in length; their flagella when present are distributed all round the bacillus; they stain with ordinary dyes, and are all Gram-negative; they are all facultative anaerobes; their optimum temperature is about 37° C.; in growth characters on ordinary media they tend to resemble one another, and generally they do not liquefy gelatin, although certain types allied to B. coli have been recognised with the property of gelatin liquefaction (*vide infra*); they show wide differences in their actions on sugars, and a primary classification of the group is based on the fact that while B. coli produces acid and gas from lactose, none of the typical pathogenic members have an effect on this sugar;

in the ultimate differentiation of the organisms *in vitro* immunity reactions are of essential importance.

The designation Bacillus coli is now well recognised as a generic term applicable to a variety of types presenting well-marked common characters though differing in biochemical reactions and in certain other features. The B. coli communis (Escherich) referred to above represents only one type. It is convenient to describe the group as a whole, indicating the differences among the more important types.

Bacillus Coli (*Escherichia*).—*Morphological Characters.*—These are best seen in young bouillon or agar cultures. The bacillus is usually 2–4 μ long and about 0·5 μ broad; longer forms up to 8 or 10 μ are not infrequent, and short cocco-bacillary forms are also noted (Fig. 104). Motility varies with different types and under different growth conditions in the same strain. The organism may stain somewhat faintly with watery dyes, but is readily demonstrated with dilute carbol-fuchsin (1 : 10); it is Gram-negative. In older cultures the bacillary protoplasm may be vacuolated and the organism may appear swollen. By appropriate staining the motile varieties can be shown to possess flagella springing from all round the organism, varying in number and rather short.

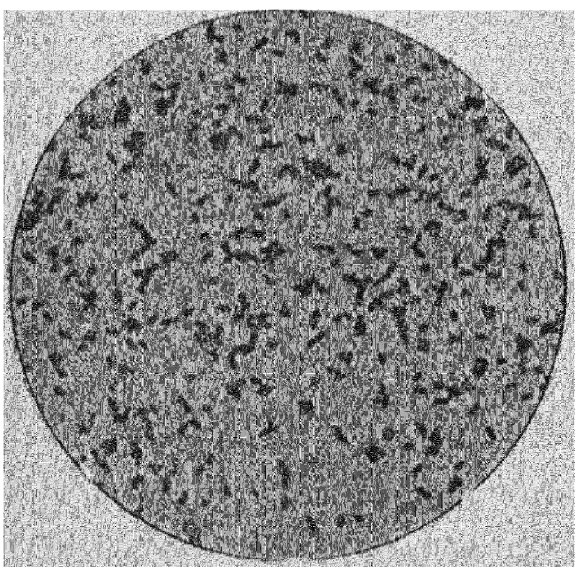


FIG. 104.—Bacillus coli. Film preparation from a young culture on agar. Stained with weak carbol-fuchsin. ×1000.

Culture Reactions on Ordinary Media.—On *sloped agar* a somewhat dense, glistening, white, or brownish-white growth occurs along the stroke of inoculation. In *stab cultures in peptone gelatin* an abundant film-like growth takes place on the surface, and there is a whitish or brownish-white line along the stab without liquefaction of the gelatin. In *bouillon*, B. coli produces a uniform turbidity. On *agar plates* the surface colonies are somewhat large, and, it may be, irregular in outline, but the deep colonies are smaller and lenticular in shape and

under a low power of the microscope appear rather dense to transmitted light. Colonies vary in size, thickness, and opacity among different strains, and in certain cases a mucoid or viscid character is observed. In fact, certain types are characterised by their large, slimy, or mucoid colonies (*e.g.* B. lactis aerogenes) (*vide infra*). On *potato*, in forty-eight hours, there is a distinct film of growth usually of a brownish tint, with a moist surface, which rapidly spreads and becomes thicker. The appearance on potato, however, varies much with the different strains and also with the reaction of the potato.

Culture Reactions on Special Media.—Various media have been used for the appreciation of special characters in the B. coli group. These reactions depend upon the capacity of the organism to originate chemical changes in a variety of substances.

A. *Fermentative Reactions on Carbohydrates.*—B. coli shows great powers of splitting up carbohydrates with the formation of acids, especially lactic acid, and gases, chiefly carbon dioxide and hydrogen. Certain types, however, classified as *B. coli anaerogenes* (*vide infra*) ferment carbohydrates without gas production.

Fermentation of Sugars.—As stated on p. 62, peptone water or casein digest with an indicator, *e.g.* litmus or neutral red, in Durham's tubes is used, the sugar to be employed being added in the proportion of $\frac{1}{2}$ to 1 per cent. The fermentative capacities of the B. coli are very wide and varied. The common types produce acid and gas in glucose, lactose, lævulose, galactose, maltose, mannite. Certain varieties ferment saccharose, dulcite, adonite, inulin, and even the benzol substance inosite (see table of sugar reactions, p. 442). Certain glucosides may also be fermented, *e.g.* salicin, arbutin. In cultures in gelatin made from fresh meat sometimes bubbles of gas appear from the fermentation of the dextrose present in the meat (Fig. 108 C.).

The reactions of B. coli in some media other than simple sugar solutions likewise depend on sugar fermentation.

Curdling of Milk.—If the B. coli be grown in milk, *e.g.* litmus milk, acid is produced from the lactose present which further curdles the milk. If litmus milk be used, the acid reaction appears to be permanent when growth is allowed to go on for some days.

Measuring of Gas Formation.—As has been said; the gases produced by B. coli in fermenting sugars are chiefly carbon dioxide and hydrogen. Many observers have attached considerable importance, first, to the amount of gas formed from a given quantity of

glucose in a given time, and, second, to the ratios of the two gases to one another, in such a fermentation. For the observation of this, MacConkey recommended the following method : fermentation tubes (p. 65, Fig. 12, *c*), with the closed limb graduated, containing 2 per cent. peptone and 1 per cent. glucose in tap water, are inoculated and incubated for forty-eight hours at 37° C. The tube is allowed to cool and the total amount of gas noted. The bulb is then filled with 2 per cent. sodium hydrate solution, the opening closed with the thumb and thoroughly shaken. After the gas has been collected in the closed arm the thumb is removed and the ratio of the hydrogen left to the original gas volume is read off.

Voges and Proskauer's Reaction.—This is a reaction which is not given by the common types of B. coli, but as it occurs with certain members of the coli group (see table) it may be described here. It also depends on carbohydrate fermentation and is due to the formation of acetyl-methyl-carbinol from glucose. A glucose peptone solution tube is inoculated and incubated for three days. A strong solution of caustic potash is added and the tube allowed to stand for several hours at room temperature. A red fluorescent colour is produced, causing the medium to resemble a weak solution of eosin.

B. *Production of Indol.*—The B. coli produces indol in peptone water. The methods have been given on page 66, and for the detection of the reaction the use of Ehrlich's rosindol test is preferable. Two peptone tubes should always be inoculated, and if the reaction is not obtainable in one after two or three days' growth, the other should be incubated for six to seven days and then tested. Where a faint reaction is obtained, it is well to corroborate the presence of indol by dissolving the rosindol out with amyl-alcohol as described.

C. *Action on Neutral Red.*—When B. coli is grown in neutral-red lactose bouillon, a rose-red colour, the effect of the lactic acid upon the dye, is at first seen. Frequently this is succeeded by the appearance of a green fluorescence due to a direct action of the organism upon the dye, of the nature of a reduction effect This is evidenced by the fact that the neutralisation of the lactic acid by an alkali does not lead to a reproduction of the original tint in the indicator. The degree of change, however, varies, the important factors being the percentage of sugar, the reaction, and the strain of the bacillus used.

D. *Reduction of Nitrates.*—The B. coli is generally capable of reducing nitrates to nitrites. For this test, Savage recommended the use of a medium made by dissolving 10 grams of peptone in 1 litre of ammonia-free distilled water, and adding 2 grams of nitrite-free potassium nitrate. The medium is filtered, tubed, and sterilised for half an hour on three days. Tubes are inoculated and incubated for forty-eight hours, the formation of nitrites being now tested for by Ilosvay's method. The following solutions are required : (*a*) sulphanilic acid, 0·5 gram dissolved in 150 c.c. dilute

acetic acid (s.g. 1·04) ; (*b*) 1 gram α-naphthylamine is dissolved in 22 c.c. of water, the solution filtered, and 180 c.c. dilute acetic acid added. In using the test, 2 c.c. of each of these solutions is added to 10 c.c. of culture. If reduction of the nitrates has occurred, a rose-pink colour should develop almost immediately. It is to be noted that the pink colour first produced sometimes disappears as it is formed or on shaking ; in such a case further portions of the two reagents in equal quantities should be added.

E. *The Methyl-Red Reaction.*—This depends on the limiting hydrogen-ion concentration attained by growth in a standard glucose peptone medium containing a standard amount of dipotassium phosphate, and has been utilised, along with the Voges-Proskauer reaction, in classifying coliform bacilli isolated from water supplies. The common types found in excreta and sewage produce a high concentration, along with a negative Voges-Proskauer reaction. Methyl-red added to the culture is used as an indicator of the resulting hydrogen-ion concentration. Organisms producing high and low hydrogen-ion concentrations are described as "Methyl-Red Positive" and "Methyl-Red Negative" respectively. For demonstrating the reaction, the organism is grown for a few days in a peptone water medium containing 0·5 per cent. peptone, 0·5 per cent. glucose, 0·5 per cent. dipotassium hydrogen phosphate ; 5 drops of a methyl-red solution (0·1 gram in 300 c.c. alcohol made up to 500 c.c. with distilled water) are added to the culture and the resulting colour noted : a red colour indicates a "positive," a yellow colour a "negative" reaction.

The neutral-red reaction and the reduction of nitrates are of relatively little importance in the differentiation of types. The indol reaction and that of Voges and Proskauer are now recognised as essential tests in the investigation of B. coli strains.

Agglutination Reactions of B. coli.—A study of the agglutination reactions of this group with the sera of animals immunised with various strains, has shown an extremely limited specificity of the agglutinin, restricted practically in its action to the individual strain used for immunisation (Mackie).

Isolation of the B. Coli.—In the case of abscesses or infection of the kidney or bladder, etc. (p. 250), the isolation of the organism is easily accomplished by inoculating plates of MacConkey's bile-salt lactose neutral-red agar (p. 58) with the pus or centrifuged deposit from urine. The colonies are usually characterised by their rose-pink colour. When the organism is present along with other bacteria, as in the case of water, sewage, etc., a bile-salt medium is also to be recommended, as it tends to inhibit the growth of organisms except those belonging to the coli-typhoid group. Where there is a mixed infection with other pyogenic organisms, *e.g.* staphylococci or streptococci, for diagnostic purposes cultures should be made also on ordinary nutrient agar to obtain a satisfactory growth of the other organisms present as well as B. coli.

The Recognition of Typical B. Coli.—The work on B. coli, especially in relation to its occurrence in water, has revealed the existence of a very large number of varieties of the organism. These differ from one another in certain biological characters, *e.g.* motility, fermentation reactions with sugars, indol formation, etc. (*vide infra*). Considerable difference of opinion exists as to what characters are to be looked upon as type characters, *i.e.* characters shared by the greatest number of varieties isolated.

Two standards may be alluded to. First, that of an English Committee which reported in 1904 on the standardisation of methods for the bacterioscopic examination of water. According to this, the B. coli is a small, motile, non-sporing bacillus, capable of growing at 37° C., Gram-negative, never liquefying gelatin, producing clot and permanent acidity of milk within seven days at 37°, fermenting glucose and lactose, with, in both, acid and gas formation—subsidiary characters being the formation of indol, the formation of a thick yellowish-brown growth on potato, production of fluorescence in neutral-red media, reduction of nitrates, and fermentation of saccharose. A similar American Committee looked upon the typical organism as a non-sporing bacillus, motile, fermenting dextrose-broth, with the formation, in the closed limb of the fermentation tube, of about 50 per cent. of gas, of which about one-third is carbon dioxide, causing acid and clot in milk in forty-eight hours, not liquefying gelatin, producing indol and reducing nitrates. These two standards differ in the fact that the English Committee laid less weight on indol formation and the reduction of nitrates.

It may be said that, in addition to these characters, the commonest types of lactose fermenters from the human and animal intestine ferment dulcite or both dulcite and saccharose, have no effect on adonite, inulin, and inosite, produce indol, and fail to give the Voges and Proskauer reaction. (*Vide* also Methyl-Red Reaction, *supra*.)

Varieties of B. Coli

From work done not only with bacteria isolated from pathological conditions, but in connection with the bacteriology of water, milk, and fæces, it has been found that a very large number of organisms exist which have the capacity of fermenting glucose and lactose, but which, when further investigated, present individual differences. Much has been done in attempting to differentiate these so-called "lactose-fermenters" from one another. Here the work of MacConkey may be taken as constituting one of the best attempts at such further classification. MacConkey showed that certain of the tests originally applied to the lactose-fermenters in reality gave little information, *e.g.* the growth in litmus whey, observation of which only corroborates what is observed with litmus milk; observation of fluorescence in neutral-red lactose media (on account of the inconstancy of the occurrence of this change among the lactose fermenters, and from the fact that many other bacteria also produce it); the reduction of nitrates—this appears to be a common pro-

perty of nearly all the members of the group ; observation of differences in the naked-eye or low-power appearances of growths on gelatin—these are very inconstant, and different colonies of the same organisms may show different appearances. On the other hand, important information may be obtained by the observation of the indol and Voges and Proskauer reactions (p. 400). With regard to sugars MacConkey claimed that in the differentiation of the lactose-fermenters, the only sugars necessary are saccharose, dulcite, adonite, inulin, and inosite. By means of these, a preliminary classification could be made from the actions on cane-sugar and dulcite, and four groups were constituted : I. Organisms not affecting either cane-sugar or dulcite, *e.g.* B. acidi lactici (Hüppe). II. Organisms having no action on cane-sugar, but fermenting dulcite, *e.g.* B. coli communis (Escherich). III. Organisms fermenting both cane-sugar and dulcite, *e.g.* the type of organism now frequently designated B. coli communior. IV. Organisms fermenting cane-sugar, but having no action on dulcite. Group IV. was further subdivided into sub-group 1, in which was no liquefaction of gelatin and an absence of the Voges and Proskauer reaction ; 2, no liquefaction of gelatin, presence of Voges and Proskauer's reaction (bacillus lactis aerogenes) ; 3, liquefaction of gelatin, presence of Voges and Proskauer's reaction (bacillus cloacæ) ; 4, liquefaction of gelatin and production of a yellow pigment.

In his later work on the B. coli group, MacConkey classified the lactose-fermenters in further detail according to the following criteria : motility, fermentation of dulcite, saccharose, adonite, inulin, inosite, the formation of indol, the Voges and Proskauer reaction, and liquefaction of gelatin. This classification elicited the fact that certain biological types were specially prevalent in the fæces of man and animals ; the type designated No. 71 ~~which~~ corresponds to the organism now spoken of frequently in bacteriological literature as *B. coli communior* (*Escherichia communior*), the *B. coli communis Escherich* (*Escherichia coli*), and *B. vesiculosus*. (See table of characters, p. 442.)

With regard to the type strains, a few words may be added. The original B. coli communis of Escherich was isolated from the intestine of newly born infants in connection with the first appearance of bacteria in the alimentary tract. About the same time, an organism now known as the B. neapolitanus was obtained by Emmerich in an outbreak of choleraic disease in Naples, and this organism was looked upon as identical with Escherich's bacillus, but it fermented saccharose, on which Escherich's had no effect. The B. acidi lactici of Hüppe was stated by this observer to be the chief cause of the souring of milk. It is now known that a large number of organisms of the same type, but differing in cultural characters, are concerned in this process, and, as a matter of fact, MacConkey found the presence of the classical strain to be relatively infrequent in milk. The B. lactis aerogenes was originally described by Escherich, in connection with his work on the bacteriology of the intestine in children, as an organism differing from the ordinary milk-souring bacteria by its producing gas from milk in the absence of air. Within recent years it has attracted attention chiefly from its apparently being closely allied to the pneumobacillus of Friedländer. Like the latter, this organism is stated when injected into animals to appear in a capsulated form.

The work on this group has shown clearly that the prevalent and characteristic coliform types found in fæces present the following common positive or negative characters : fermentation of lactose with gas production, production of indol, absence of fermentation of inosite, absence of gelatin liquefaction : the recognition of these characters becomes of great practical importance in the examination of water for " typical " B. coli where these organisms are taken as an index of fæcal pollution.

It has been shown by Mackie in a study of the biological relationship of the different types of B. coli by means of the group reaction with complement-fixing antisera, that indol formation and inosite fermentation constitute important criteria in the grouping of these organisms, and that the fermentation of the various carbohydrates, even lactose fermentation, is of less significance. Thus, non-lactose-fermenters which ferment glucose and certain other carbohydrates and correspond in their general characters with B. coli, but are not referable to the specific pathogenic groups (typhoid, paratyphoid, etc.), fall to be classified with the coliform bacilli, and it is well known now that certain coliform strains only ferment lactose slowly or after mutation (*vide* p. 27).

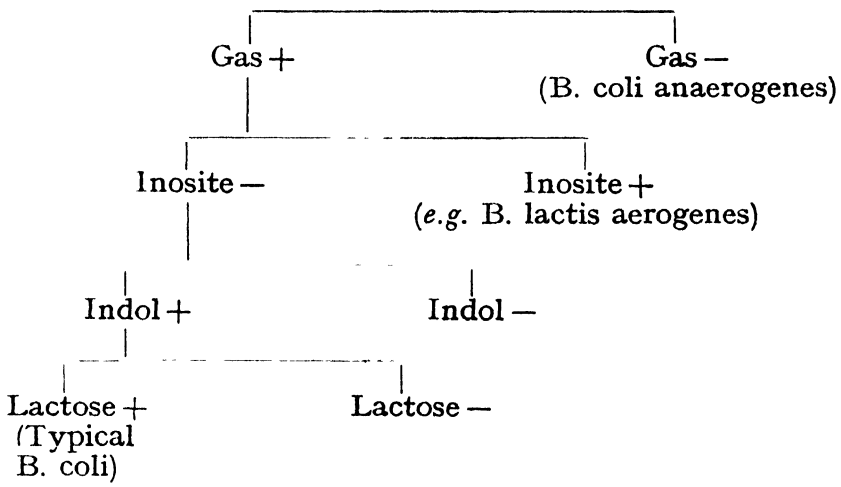
The separate grouping of the inosite fermenters is supported by the fact that these organisms show other common characters—non-motility, a mucoid capsule, large slimy viscid colonies which constitute a characteristic feature of their growth, fermentation of lactose and adonite. It is to this sub-group that the Voges and Proskauer reaction pertains, though it is not a character of all strains. The B. lactis aerogenes exemplifies these organisms.

The question arises as to whether gelatin-liquefying, Gram-negative bacilli which correspond in other characters to B. coli types are to be included in this biological group. Among the lactose-fermenters classified by MacConkey certain gelatin liquefiers were represented, *e.g.* B. cloacæ, etc., and other authorities (Prescott and Winslow) have accepted this reaction as one of the possible characters of the group.

From our present knowledge of the aerobic intestinal bacilli it would appear more rational from the purely biological standpoint to recognise a large class of intestinal bacilli having the common characters : aerobic, Gram-negative, non-sporing, growing at 37° C., fermenting glucose with or without gas production, not liquefying gelatin ; comprising certain specific pathogenic organisms specially designated, and identified by cultural and serological characters, *e.g.* B. typhosus, etc., and a large number of saprophytic, though potentially pathogenic, varieties which can be generally classified into different types according to their cultural characters. The term B. coli (if it is to be used at all) in its widest application would thus become referable to those members of the class which do not possess specific pathogenic properties, irrespective of certain cultural characters including lactose fermentation. The term " typical B. coli " may be used to designate those types (fermenting lactose, producing indol, etc.) which are most prevalent in the intestine, and therefore undoubted indicators in water of recent sewage pollution. The only justification for recognising a special group of " lactose-fermenters " depends on the statistical fact that these types are most prevalent in fresh animal excreta, but it is

questionable if the statistical basis can be used for a biological classification.

The coliform bacilli can thus be broadly classified according to the following system which allows of the inclusion in the group of the various atypical strains, *e.g.* various non-lactose fermenters, the so-called "paracolon" types, etc., which possess no specific pathogenic properties :



Variation and Mutation in the B. coli group has been dealt with in Chapter I.

Pathogenic Properties of the B. Coli.—In man, the B. coli has been found as the only organism present in various suppurative conditions (see Chapter VII.), especially in connection with the intestine (*e.g.* appendicitis) and about the urinary tract. In the latter, it is also responsible for catarrhal conditions in the pelvis of the kidney and in the bladder, these being more common in the female, and frequently presenting chronic characters. B. coli infection of the pelvis of the kidney may be the sequel to cystitis, *i.e.* an ascending infection, but cases are not infrequent in which the organism has reached the kidney either by the blood stream or, as has been suggested, by communicating lymphatic channels from the bowel.

Many interesting problems arise in regard to the pathogenicity of B. coli. Under ordinary conditions this organism exists as a harmless commensal in the bowel of all the mammalian animals, and it is present usually in enormous numbers. No injurious influence is exerted normally on the intestinal mucosa. It seems probable, however, that small numbers may in the healthy person gain access to the lymphatics and even the blood stream ; these are rapidly eliminated, but in certain conditions of lowered resistance may become established in certain tissues, *e.g.* pelvis

of the kidney. Obviously the healthy intestinal mucosa presents a high degree of local immunity to these organisms, whereas other tissues are less resistant.

The question also arises as to the rôle of B. coli in various pathological conditions of the intestine initiated by specific pathogens, *e.g.* B. typhosus, B. dysenteriæ, etc. Sanarelli found that B. coli isolated from typhoid stools was much more virulent for laboratory animals than when isolated from the stools of healthy persons. Coliform bacilli probably play the part of a secondary infecting organism when the mucous membrane is damaged or ulcerated by some other virus, *e.g.* in typhoid fever, in amœbic and bacillary dysentery, etc. Certain types may be more active in this respect than others. Attention has been drawn to the fact that in bacillary dysentery concomitant or secondary infections occur particularly with various non-lactose-fermenting strains, *e.g.* B. Morgan No. 1 and related organisms, B. paracolon types (*vide infra*).

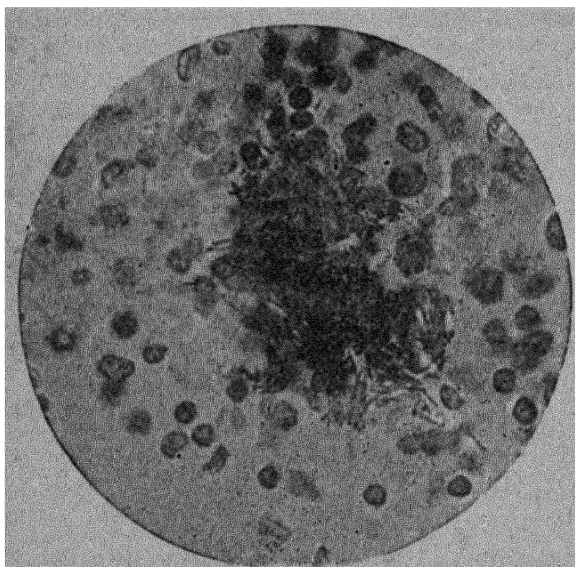


FIG. 105.—A large clump of typhoid bacilli in a spleen. The individual bacilli are only seen at the periphery of the mass. (In this spleen enormous numbers of typhoid bacilli were shown by cultures to be present in a practically pure condition.) Paraffin section; stained with carbol-thionin-blue. ×500.

Bacillus Typhosus (*Eberthella typhi*).—*Microscopical Appearances.*—As observed in pure culture and in the tissues, the individual organisms are straight, cylindrical, or rod-shaped structures, with rounded ends, measuring on an average 2–4 μ long and 0·5 μ in thickness. Frequently in culture filamentous forms are noted, even 10 μ or more in length. In culture the bacilli occur singly and in pairs end to end. In tissues, *e.g.* in a Peyer's patch, spleen, liver, or mesenteric gland, they are found in relatively large clumps (*vide* Fig. 105).

For their demonstration in tissue sections, carbol-thionin-blue may be used (*vide* p. 105), or prolonged staining with Löffler's methylene-blue (half an hour), the sections being dehydrated

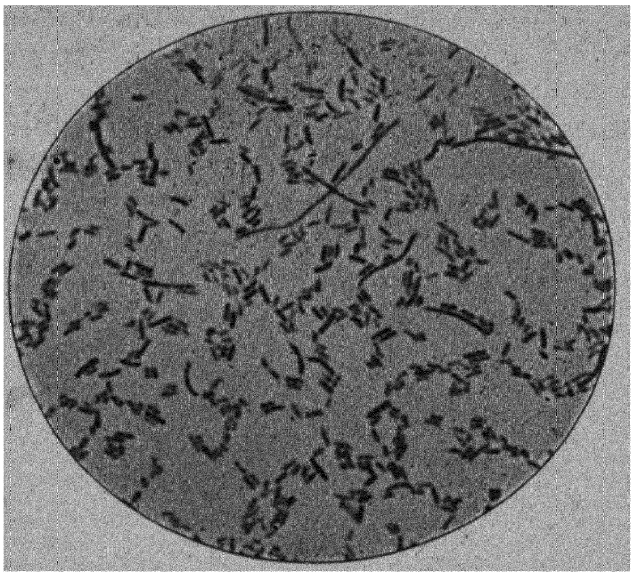


FIG. 106.—Typhoid bacilli, from a young culture on agar, showing some filamentous forms.
Stained with weak carbol-fuchsin. ×1000.

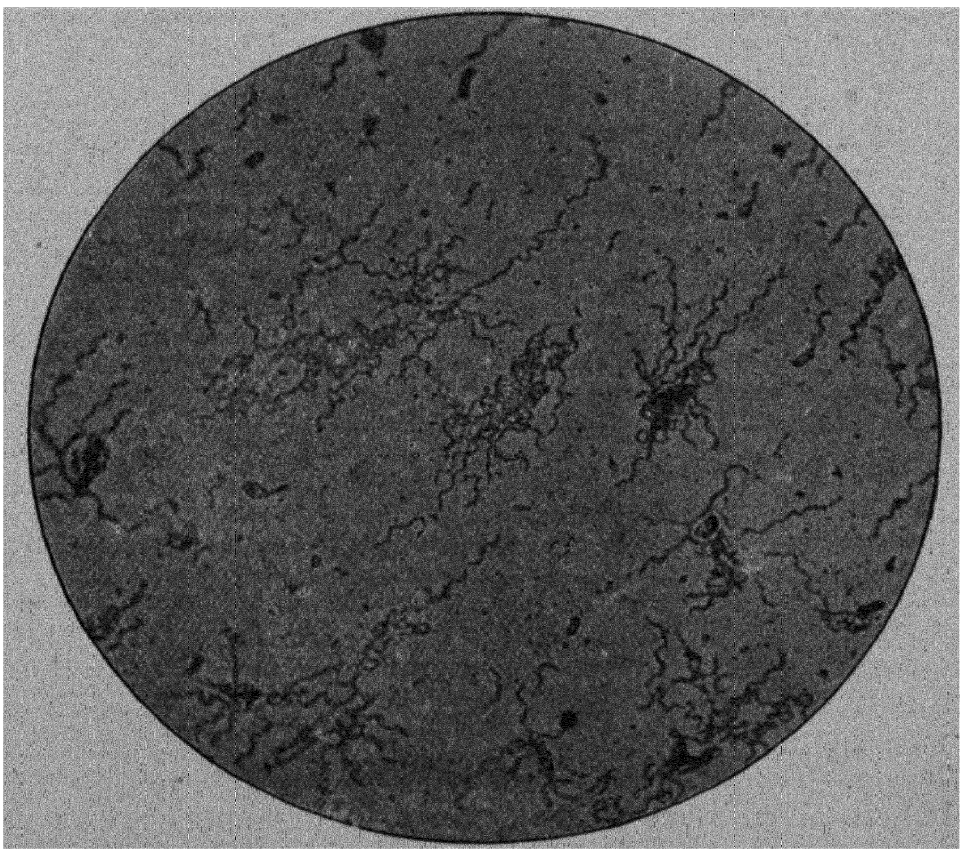


FIG. 107.—Typhoid bacilli, from a young culture on agar, showing flagella. See also Plate III., Fig. 15.
Stained by Van Ermengem's method. ×1000.

and cleared with aniline oil (*vide* p. 104). The B. typhosus is Gram-negative.

Motility.—In hanging-drop preparations the bacilli are found to be actively motile. The smaller forms have a darting or rolling motion, passing quickly across the field; whilst some show rapid rotatory motion. The filamentous forms have an undulating or serpentine motion, and move more slowly.

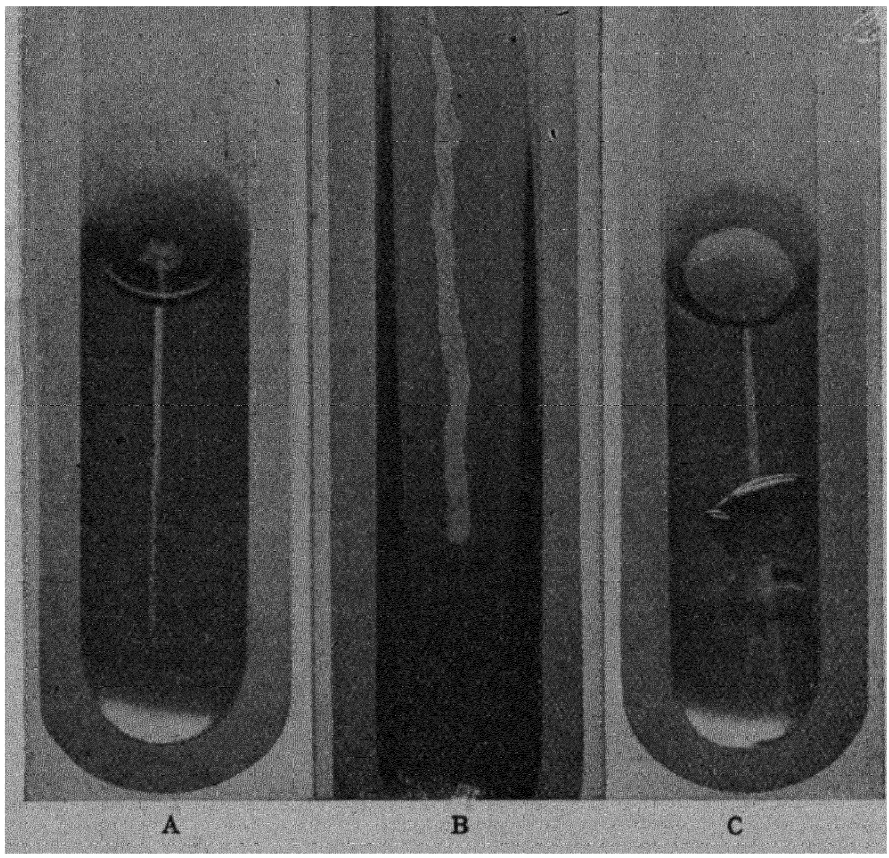


FIG. 108.

A. Stab culture of the typhoid bacillus in gelatin, five days' growth.
B. Stroke culture of the typhoid bacillus on gelatin, six days' growth.
C. Stab culture of the bacillus coli in gelatin, nine days' growth; the gelatin is split in the lower part owing to the formation of gas.

Hanging-drop preparations ought to be made from agar or broth cultures not more than twenty-four hours old. In older cultures the movements are less active.

Flagella.—On being stained by the appropriate methods (*vide* p. 112), the bacilli are seen to possess many long wavy flagella which are attached all along the sides and to the ends (Fig. 107). They are more numerous, longer, and more wavy than those of the B. coli.

Appearances of Cultures.—To grow the organism artificially from post-mortem cases it is best to isolate it from the spleen (for method, see p. 149), or from the bile in the gall-bladder, as it exists in these situations in greater numbers than in the other organs, and may be the sole organism present even some time after death. Agar or preferably MacConkey plates may be employed (*vide* p. 58). The obtaining of pure cultures from cases during life will be dealt with in detail later (p. 437). On culture media the growths are visible after twenty-four hours' incubation at 37° C. On MacConkey plates the colonies are small and colourless (cp. B. coli).

Characters of Culture.—Generally speaking, on artificial media growths of the B. typhosus appear less dense than those of the B. coli.

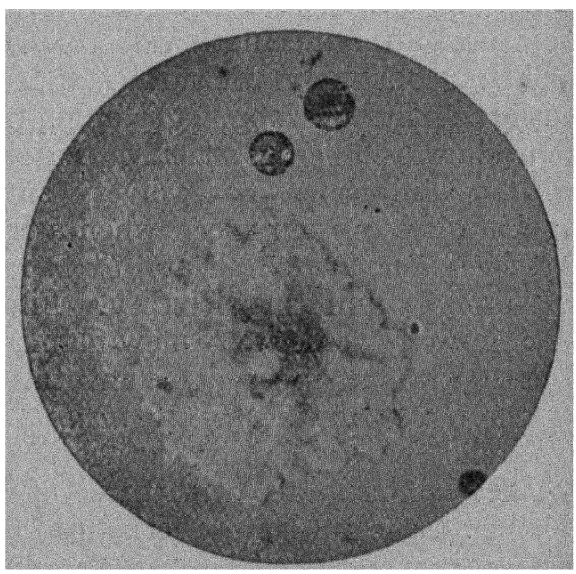


FIG. 109.—Colonies of the typhoid bacillus (one superficial and three deep) on a gelatin plate. Three days' growth at room temperature. ×15.

In stroke cultures on *agar* there is a bluish-grey film of growth, with fairly regular margins, but without any characteristic features. This film is moist, loosely attached to the surface, and can be easily scraped off. Colonies on nutrient agar after twenty-four hours' incubation are small, clear, circular discs, but if growth is allowed to continue for a few days they enlarge considerably, become film-like with a wavy margin, a raised centre, and often radial ridges; this type of colony has been likened in shape to a vine leaf. Stab cultures in *peptone gelatin* yield the following appearance: on the surface of the medium growth spreads outwards from the puncture as a thin leaf-like film, with irregularly wavy margin (Fig. 108, A); it is semi-transparent and of bluish-white colour; ultimately this surface growth may reach the wall of the tube; not infrequently, however, the surface growth is less marked; along the stab there is an opaque whitish line of growth, of finely nodose appearance; there is no liquefaction of the medium. On gelatin, surface colonies are more transparent than those on agar but present similar features (Fig. 109).

Arkwright has found that certain strains of B. typhosus and other members of the group—B. paratyphosus B, B. dysenteriæ (Shiga and Flexner), B. enteritidis (Gaertner), etc.—give two types of colonies on plates, these being rough (R) and smooth (S) respectively (Fig. 110). Growths from the R colonies are spontaneously agglutinable in normal saline (though forming permanent suspensions in weak saline) and form in bouillon a sediment and surface scum with clear fluid, in these respects differing from the S growths. The two types differ also in their antigenic structure and their agglutination reactions, but this is less marked with the B. typhosus than with B. dysenteriæ. The properties of the two types are maintained in subcultures.

Growth on Potato.—For several days (at incubator temperature) after inoculation there is apparently no growth. If looked at obliquely, the surface appears wet, and if it is scraped with the platinum loop, a glistening track is left : a film preparation shows numerous bacilli. Later, however, a slight pellicle with a dull, somewhat velvety surface may appear. These appearances are only seen when a fresh potato has been used.

In *bouillon* incubated at 37° C. for twenty-four hours there is simply a uniform turbidity. Film preparations made from such growths sometimes show filamentous forms of considerable length without apparent segmentation.

On *MacConkey's bile-salt neutral-red lactose agar.*—Colonies resemble those on ordinary agar. They remain colourless or " pale " as compared with those of B. coli (*vide* p. 401), owing to the absence of lactose fermentation.

Conditions of Growth, etc.—The optimum temperature of the typhoid bacillus is about 37° C., though it also flourishes well at room temperature. It will not grow below 15° C., or above 41° C. Its powers of resistance correspond with those of most non-sporing bacteria. It is killed by exposure for ten minutes at 56° C., or almost instantaneously at 100° C. Typhoid bacilli kept in distilled or in ordinary tap water have usually been found to be dead after three weeks (Frankland).

Biological Reactions.—The growth of the typhoid bacillus on certain special media facilitates its being differentiated from the B. coli and the other members of the coli-typhoid group by biochemical reactions. (See Table, p. 442.)

The tests with sugars are important. The typhoid bacillus produces acid *without gas* in glucose, lævulose, galactose, and mannite, and as a rule in maltose, but originates no change in lactose, saccharose, or dulcite ; in the last, however, late acid formation may occur. Further, there is no curdling of milk,

although slight acid production occurs; in a variable time the acid change may be succeeded by alkali production. Under ordinary circumstances, the typhoid bacillus is incapable of producing indol in peptone solution, and does not alter neutral red in lactose bouillon.

Strains of the typhoid bacillus have been noted which differ in certain biochemical reactions. Thus two groups have been described which differ in regard to the fermentation of xylose, one producing rapid fermentation, the other acting on this substance only after several days.

The identity of the typhoid bacillus can be conclusively established by means of agglutination reactions which will be treated of later (p. 441).

The Pathology of Typhoid Fever.[1]—The inflammation and ulceration in the *Peyer's patches and solitary glands of the intestine* are the central features. In the early stage there is an acute inflammatory condition, infiltration with mononuclear cells, and attended sometimes with small hæmorrhages. At this period the typhoid bacilli are most numerous in the patches, groups being easily found between the cells. The subsequent necrosis may be due to the action of the toxic products of the bacilli, which, however, gradually disappear, though they may still be found in the deeper tissues and at the spreading margin of the necrosed area. They also occur in the lymphatic spaces of the muscular coat. The number of the ulcers arising in the course of a case bears no relation to its severity. Small ulcers may occur in the lymphoid follicles of the large intestine.

The *mesenteric glands* corresponding to the affected part of the intestine are usually enlarged, sometimes to a very great extent, the whole mesentery being filled with glandular masses. In such glands there may be acute inflammation, and occasionally necrosis in patches occurs. An outstanding feature of the lesions is the extensive proliferation of the endothelial cells along the lymph sinuses and paths, attended by aggregations of mononuclear leucocytes. In other words, the reaction is of the *macrophage* type, and few polymorpho-nuclear leucocytes are present as a rule. Sometimes on section the glands are of a pale-yellowish colour, the contents being diffluent and consisting largely of leucocytes. Typhoid bacilli may be isolated

[1] In the succeeding sections dealing with the pathology of typhoid fever, the pathogenesis of the typhoid bacillus, carriers, and epidemiology, most of the facts recorded apply also to the paratyphoid bacilli (described later); enteric fever or "enterica" is a convenient general designation for both typhoid and paratyphoid infections.

both from the glands and the lymphatics connected with them but the B. coli is in addition often present.

The *spleen* is enlarged—on section usually of a fairly firm consistence, of a reddish-pink colour, and in a state of congestion. Of all the solid organs it usually contains the bacilli in greatest numbers. They can be seen in sections, occurring in clumps between the cells, there being no evidence of local reaction round them (Fig. 105). Similar clumps may occur in the *liver* in any situation, and without any local reaction. In this organ, however, there are often small foci of leucocytic infiltration, in which bacilli may not be demonstrable. The bacillus is found, often in large numbers, in the gall-bladder, where, in recovered cases, it may persist for years (*vide infra*). Clumps of bacilli may also occur in the *kidney*.

In addition to these local changes in the solid organs, there are also widespread *cellular degenerations* in the solid organs which suggest the action of toxic products.

In the *lungs* there may be bronchitis, patches of congestion and of acute broncho-pneumonia. In these, typhoid bacilli may sometimes be observed, but evidence of a toxic action depressing the powers of resistance of the lung tissue is found in the fact that the pneumococcus frequently occurs in such complications of typhoid fever.

The *nervous system* shows little change, though meningitis associated either with the typhoid bacillus, with the B. coli, or with streptococci has been observed

During the first seven to ten days of the illness, and also in relapses, the bacilli can be isolated from the *blood*. They have been found in the *roseolar spots* which occur in typhoid fever, but it cannot be yet stated that such spots are always due to the presence of the bacilli. The fact that the typhoid bacilli are usually confined to certain organs and tissues shows that they probably have a selective action.

The reaction of the body to the typhoid bacillus is markedly one of the lymphoid tissues. This is further evidenced by the blood changes, for while there is a leucopenia the lymphocytes are relatively increased in numbers. A general reaction is manifested by the appearance of bactericidal and other antibodies in the serum.

The view of the development of the disease usually taken is that the bacilli, being ingested, multiply in the intestinal tract, cause inflammation and necrosis of the lymphoid tissue, and, gaining an entrance to the general circulation, produce the bacteriæmia which we have described.

Suppurations occurring in connection with Typhoid Fever.—In a certain proportion of such suppurations the typhoid bacillus has been the only organism found. This has been the

case in subcutaneous abscesses, in suppurative periostitis, suppuration in the parotid, abscesses in the kidneys, empyema, etc., and also in a few cases of ulcerative endocarditis; suppurations due to the typhoid bacillus may be of a very chronic and recurrent nature. In the majority of cases other organisms, especially the B. coli and the pyogenic micrococci, have been obtained, the typhoid bacillus having been searched for in vain. It has, moreover, been experimentally shown, notably by Dmochowski and Janowski, that suppuration can be experimentally produced by injection in animals, especially in rabbits, of pure cultures of the typhoid bacillus, the occurrence of suppuration being favoured by conditions of depressed vitality, etc. These observers also found that when typhoid bacilli were injected along with pyogenic staphylococci, the former died out in the pus more quickly than the latter. Accordingly, in clinical cases where the typhoid bacillus is present alone, it is improbable that other organisms were present at an earlier date.

Occurrence of Gall-stones in those who have suffered from Typhoid Fever.—As has been stated, foci of bacilli occur in the liver in typhoid fever, and infection of the gall-bladder is a frequent feature of the disease. In the gall-bladder they may set up a catarrhal process (*cholecystitis typhosa*), though frequently they produce comparatively little change in the mucosa. There is evidence that the bacilli may persist in the gall-bladder for many years, and the catarrhal inflammation which they keep up leads to the formation of gall-stones. B. coli may be superadded. Typhoid bacilli have been isolated from cases of gall-stones operated on years after an attack of typhoid fever, and the bacilli have even been found within the calculi. They have also been demonstrated in chronic suppurations occurring in the gall-bladder.

Pathogenic Effects produced in Animals by the Typhoid Bacillus.—There is no disease of animals which is identical with typhoid, nor is there any evidence of the occurrence of the typhoid bacillus under ordinary pathological conditions in the bodies of animals. Attempts to communicate the disease to animals by feeding them on typhoid dejecta have been unsuccessful, and though pathogenic effects have been produced by introducing pure cultures in food, the disease has usually borne no resemblance to human typhoid. The results of subcutaneous or intraperitoneal infection are no more satisfactory. The type of disease is very different from what occurs naturally in man, and is more of the nature of an acute toxæmia due to the endotoxin present in the organisms. In such injection

experiments the results vary considerably—no doubt due to the fact that different strains of the bacillus vary much in virulence, ordinary laboratory cultures being often almost non-pathogenic. Certain workers using highly virulent strains have been able by intraperitoneal injection to produce in animals effects on the Peyer's patches, mesenteric glands, and spleen similar to those in the disease in the human subject. It has also been found by experiments in rabbits in which recently isolated cultures of B. paratyphosus A were introduced directly into the gall-bladder, that a pathological picture corresponding to that of the natural disease could be reproduced, with characteristic effects on the lymphoid patches in the intestine, the mesenteric glands and the spleen. Metchnikoff and Besredka claimed to have reproduced in monkeys a condition similar to human typhoid fever by feeding the animals with typhoid bacilli. It is specially noteworthy that in rabbits injected intravenously with typhoid cultures, the bacilli may become localised in the gall-bladder, where they persist as in the human carrier.

The Toxic Products of the Typhoid Bacillus.—Here very little light has been thrown on the pathology of the disease. There exist in the bodies of typhoid bacilli toxic substances which in artificial cultures do not pass to any great degree out into the surrounding medium; they have no specific effect. The bodies of bacteria killed by chloroform vapour are very toxic—more so than filtered cultures—and there is evidence of the release of poisons from the organisms when these undergo bacteriolysis in the animal body. Allan Macfadyen, by grinding up typhoid bacilli frozen solid by liquid air, produced a fluid whose toxic effect he attributed to the presence of the intracellular poisons.

The Immunisation of Animals against the Typhoid Bacillus.—Earlier observers had been successful in accustoming mice to the typhoid bacillus by the successive injections of small and gradually increasing doses of living cultures of the bacillus. Later, Brieger, Kitasato, and Wassermann found that the bacillus when modified by being grown in a bouillon made from an extract of the thymus gland no longer killed mice and guinea-pigs. These animals after injection were moreover immune, and it was also found that the serum of a guinea-pig thus immunised could, if transferred to another guinea-pig, protect the latter from the subsequent injection of a dose of typhoid bacilli to which it would naturally succumb. Chantemesse and Widal, Sanarelli, and also Pfeiffer, immunised guinea-pigs against the subsequent intraperitoneal injection of virulent living typhoid bacilli, by repeated and gradually

increasing intraperitoneal or subcutaneous doses of dead typhoid cultures in bouillon. Experiments performed with serum derived from typhoid convalescents indicate that it has similar *anti-bacterial* powers, but there is no evidence that it contains any *antitoxic* bodies (see chapter on Immunity). Pfeiffer, for example, found on adding serum from typhoid convalescents to typhoid bacilli killed by heat, and injecting the mixture into guinea-pigs, that death took place as in control animals which had received these toxic agents alone. Pfeiffer also found that by using the serum of immunised goats, he could, to a certain extent, protect other animals against the subsequent injection of virulent living typhoid bacilli. On trying to use the agent in a curative way, *i.e.* injecting it only after the bacilli had begun to produce their effects, he got little or no result.

General View of the Relationship of the B. Typhosus to Typhoid Fever.—1. Typhoid fever is a disease in which the lesions are centred in the intestine, but the infection is essentially a general and systemic one, and secondary effects may occur in other parts of the body. The specific association of the typhoid bacillus with the disease and its characteristic lesions has been thoroughly established by bacteriological investigation. Its almost constant presence in the blood at an early stage is specially significant of its etiological relationship.

2. The comparative failure of attempts to cause the disease in animals is of little significance. In nature animals do not suffer from this condition, and laboratory animals are also insusceptible to the infection as it occurs in man.

3. The observations on the protective power against typhoid bacilli shown to belong to the serum of typhoid patients and convalescents, and the action of such serum in agglutinating the bacilli (*vide infra*), indicate an etiological relationship between the bacillus and the disease. Additional evidence is found in the fact that vaccination with the dead bacilli (*vide infra*) has a marked effect in preventing the disease from arising in those exposed to infection, and also in lowering the mortality when the fever attacks vaccinated persons.

These facts constitute indirect but practically conclusive evidence of the causal relationship of the typhoid bacillus to the disease. Confirmation of this view is found in the fact that cases have occurred where bacteriologists have accidentally infected themselves by the mouth with pure cultures of the typhoid bacillus, and after the usual incubation period have developed typhoid fever. Several cases of this kind are on record and their significance is not affected by the fact that other

similar instances have occurred without the subsequent development of illness. These latter would be accounted for by a low degree of susceptibility on the part of the individual or to a want of pathogenicity in the cultures.

There is evidence that certain individuals are relatively insusceptible to typhoid fever. The cases of the occurrence of typhoid bacilli in the healthy intestine support this view, and it has been further shown that during an epidemic certain persons may suffer from slight intestinal symptoms with typhoid bacilli in the fæces without the disease going through its usual course. The so-called " ambulatory " cases of typhoid fever form a link between these mild infections and fully developed typhoid fever.

Typhoid Carriers.—In the great majority of cases of typhoid fever, the bacilli disappear from the fæces within from two to ten weeks of convalescence, but in a certain proportion of cases, probably about 2 to 5 per cent., evidence is found of the persistence of the bacilli for many months, and in certain cases their existence has been demonstrated even thirty and, it may be, fifty years after the attack of illness. Carriers have been arbitrarily classified as " temporary " (*i.e.* those excreting bacilli up to a year after an attack of fever) and as " chronic " (those where this period is exceeded), but the distinction is unimportant. It may be said that the majority of carriers to whom outbreaks have been traced are women. Persons in whom the carrier phenomenon is present are a constant danger to those around them, as the infectivity of the bacilli frequently remains, and during recent years the importance of such carriers has been recognised as explaining many outbreaks of the disease. The cases traceable to such an origin are of the type usually classed as sporadic. They arise amongst persons associated with carriers, especially when the latter are concerned in the preparation of food. From time to time, however, large epidemics have arisen from a carrier having contaminated a milk supply in a dairy. The site of the multiplication of the bacteria in a great many of these carriers is probably the gall-bladder (see p. 413). As has been stated, the typhoid bacilli may persist there for many years, often giving rise to gall-stones. An additional danger lies in the fact that carriers usually appear to be in perfect health or may only suffer from slight, and to them unimportant, pains in the region of the gall-bladder, it being well known that in only a proportion of patients suffering from gall-stones do severe symptoms arise. An additional factor in the carrier problem lies in the fact stated above, that apparently when certain persons ingest the typhoid bacilli, the latter may

multiply for some months in the intestinal tract without giving rise to typhoid fever. Such persons have been referred to as "paradoxical" carriers; they represent those who either are naturally insusceptible to typhoid fever or have developed immunity in consequence of a previous attack. The most serious danger to a community arises, however, from the "chronic" carrier. In certain carriers, the focus of multiplication of the typhoid bacillus may not be the bowel but the kidney, the bacilli in such cases passing out in the urine. Urinary carriers are much less common than intestinal carriers; but, unlike the latter, they occur equally in the two sexes.

The "tracking down" of a typhoid carrier constitutes an important and difficult problem. Firstly, the serum of all suspicious persons ought to be subjected to the Widal test (*vide infra*). Usually speaking, the carrier gives a positive reaction, but sometimes this is absent and sometimes is only obtained with a high concentration of the serum. Further, it has been shown in chronic carriers that the agglutinating capacity of the serum varies from time to time and sometimes may be absent. The proof of a person being a carrier lies essentially in the isolation of the typhoid bacillus from the fæces or the urine, and it is to be noted that, especially in the former, the organism is not constantly present—in certain cases months of remission have been recorded. Several explanations have been advanced to account for the facts observed, such as the occurrence of symptomless re-infections or of periodic more or less acute auto-infections from a latent focus of persistence of the bacterium, *e.g.*, in the gall-bladder. In any case, the necessity for repeated investigation of a suspected carrier is obvious. The methods to be adopted are detailed on p. 437. Much work has been directed to the question of freeing the typhoid carrier from the organism; various methods, such as intestinal antisepsis, vaccination, excision of the gall-bladder, have been tried. Hitherto success has not been obtained except as a result of operative procedures (excision of the gall-bladder and drainage of the ducts), which appear to have cured a considerable proportion of intestinal carriers. From the public health standpoint, the prevention of the presence of carriers in a population must be provided for; and in fever hospitals means ought to be taken for retaining convalescents from typhoid until the bodily discharges are free from the typhoid bacillus. This is now widely practised.

The Epidemiology of Typhoid Fever.—In civilised communities the prevalence of typhoid fever has been very markedly reduced, coincident with the substitution of central filtered

water supplies for well waters and with the improvements effected in general sanitation and especially in the removal of excreta and refuse. In certain localities, however, periodic outbreaks, often of a seasonal character, still occur. At one time these were attributed to the capacity of the typhoid bacillus to live for long periods and to multiply outside the human body. There is, however, no evidence that the typhoid bacillus can maintain a saprophytic existence though it may remain viable in sewage, water, etc., for a variable time. In water it may survive for at least some days, and even for two or three weeks, the time depending on the degree of contamination of the water with other organisms. Thus typhoid bacilli tend to persist longer in a relatively pure water than in one grossly polluted and containing large numbers of putrefactive organisms. The existence of carriers in all communities where typhoid fever occurs has, however, thrown new light on the subject and has accounted for the origin of many outbreaks otherwise obscure. In many cases survival outside the body for some time is an essential factor where a water or food supply becomes infected with material derived from a carrier, but the viability of the bacillus under saprophytic conditions is of less importance than was originally supposed, and direct and mediate contact infection plays a great part in the incidence of the disease. At the present time small outbreaks frequently originate in those who are brought into domestic contact with carriers, and larger epidemics may occur when a carrier pollutes a water or especially a milk supply. During such outbreaks secondary cases may also arise from contact with primary cases.

It is now well known that the house-fly and certain other filth-feeding insects may act as vectors of typhoid and paratyphoid bacilli, and contaminate food to which they gain access after having been in contact with human excreta. Such convection is most marked under conditions of imperfect sanitation, in which human excretal matter is freely exposed to flies, particularly in tropical and subtropical countries and also in the warm seasons of temperate climates when flies are most numerous. This affords some explanation of the seasonal incidence of enteric fever, its prevalence in ill-sanitated tropical and subtropical countries and its incidence under war conditions, particularly in campaigns waged in hot climates, as was well exemplified in the early stages of the Mediterranean campaign of the late war, when enteric fever (due mostly to the paratyphoid bacilli) caused a serious degree of invalidism and mortality.

The fly may carry excretal bacteria on the surface of its body,

wings, and legs, but typhoid bacilli may also persist in the alimentary tract of the insect after ingestion of infective excreta (Graham Smith and others). Food is thus contaminated by the insect as a result of its regurgitation and defæcation. In a series of experiments, in which captive flies were artificially infected with typhoid and paratyphoid bacilli and after varying intervals allowed to walk about on a plate of MacConkey's medium which was then incubated, it was found that they remained infective for as long as six days (Mackie). Colonies of the bacilli developed on the plate in circular groups suggestive of their originating from defæcated or regurgitated material.

Occasional outbreaks of enteric fever and sporadic cases have been traced to oysters and other shellfish, uncooked vegetables such as water-cress, lettuce, etc., contaminated from sewage or human excreta. It has been shown that typhoid bacilli may persist in living oysters for a considerable time.

The Serum Diagnosis of Typhoid Fever—Widal Reaction.—While the only conclusive method of establishing the diagnosis of a typhoid infection is the isolation of the organism (*vide* p. 437), the indirect proof of the infection by serum diagnosis has been extensively used in routine laboratory work. The method of carrying out this test has been described on p. 123. It depends on the presence in the patient's blood serum of agglutinins for the typhoid bacillus. If the microscopic method (*vide* p. 124) is used, *complete* clumping by a serum in a dilution of 1 : 30 within half an hour, may be regarded as of diagnostic significance, provided that the patient has not been inoculated against typhoid, and has not previously suffered from the disease. Suspicion should be entertained as to the diagnosis if agglutination occurs only in a higher concentration, or after a longer time. The naked-eye method, however, is to be preferred as more reliable since the test is carried out on a strictly quantitative basis and the highest dilution in which agglutination occurs, *i.e.* the "end-titre," can be determined. If necessary, parallel tests may be carried out with B. paratyphosus A and B (*vide* p. 424). Owing to the fact that normal serum may agglutinate the typhoid bacillus in low dilutions, it is necessary to base the diagnostic interpretation of results on what may be spoken of as the "criterion" of a positive reaction. It may be concluded from a study of such "normal serum effects" that if marked agglutination occurs in a dilution of 1 : 100 with average cultures of B. typhosus by the macroscopic method in a person not vaccinated against typhoid bacilli, the result may be regarded as diagnostic.

All strains do not give uniformly the same results, though it is not known on what this difference of susceptibility depends. A strain must therefore be selected which gives the best result in the greatest number of undoubted cases of typhoid fever, and which gives as little reaction as possible with normal sera or sera derived from other febrile diseases. This latter point is important, as some strains react very readily to non-typhoid sera. Again, care must be taken as to the *state of the culture* used. The suitability of a culture may be impaired by varying the conditions of its growth. Dreyer's standardised bacillary suspensions are of great value (p. 125).

The reaction given by the serum in typhoid fever usually begins to be observed about the seventh day of the disease, though occasionally it has been found as early as the fifth day, and sometimes it does not appear till the third week or later. Cases are also met with in which there may be a complete absence of agglutinin response even in convalescence. Usually it becomes gradually more marked as the disease advances, and it is still given by the blood of convalescents. Further, it may persist for several months or longer after recovery. As a rule, up to a certain point, the reaction is more marked where the fever is of a pronounced character, whilst in the milder cases it is less pronounced, but this is not invariably the case. In certain grave cases, however, the reaction has been found to be feeble or almost absent.

The reaction is obtained not only with living bacilli, but also with bacilli that have been killed by heating at 60° C. for an hour—if a higher temperature be used, sensitiveness to agglutination is impaired. Besides the blood serum, it has been found that the reaction is given in cases of typhoid fever by pericardial and pleural effusions, by the bile and by the milk. It may here also be mentioned that a serum will stand exposure for an hour at 57° C. without having its agglutinating power much diminished. Higher temperatures, however, cause the property to be lost.

Owing to the fact that the development of the specific agglutinin is progressive during the illness, if, on first testing, agglutination is absent or occurs only in low dilutions, *e.g.* below 1 : 100 in the case of the macroscopic method, the test should be repeated. In this way a more conclusive result may be obtained. A series of tests carried out at intervals may elicit a "rising" end-titre which is, of course, of the highest diagnostic significance.

In cases previously vaccinated with the typhoid bacillus, a serious fallacy may be introduced into the ordinary diagnostic test when applied to such persons developing an illness suspected

to be a typhoid infection. Thus in healthy persons vaccinated from seven to fourteen months previously, the serum may agglutinate the typhoid bacillus in dilutions of 1 : 200 and even in some cases in higher dilutions (Martin and Upjohn). Again, in those who have been vaccinated with the typhoid bacillus, a subsequent paratyphoid infection may lead to an increase in the "post-vaccine" typhoid agglutinin and high-titre reactions may occur with the typhoid bacillus as well as with the causal organism (Mackie and Wiltshire). Other infections, *e.g.* various febrile illnesses, may produce a similar effect, and in vaccinated persons the practical value of the reaction is reduced to a minimum unless agglutination occurs on first or subsequent testing in high dilutions, *e.g.* 1 : 1000 or over. This question will be referred to further in connection with the paratyphoid infections.

Vaccination against Typhoid.—The principles of the immunisation of animals against typhoid bacilli were originally applied by Wright and Semple for prophylactic purposes. The method of preparing the vaccine has been described on p. 139. Two doses have usually been given, separated by an interval of ten days, the first consisting of 500 million bacilli and the second of 1000 million. The effects of the first injection are some tenderness locally and in the adjacent lymphatic glands, and, it may be, local swelling, all of which come on in a few hours, and may be accompanied by general malaise and a rise of temperature, but the illness is transient. During the next ten days the blood of the individual begins to manifest, when tested, an agglutination reaction, and further, Wright found that usually after the injection there is a marked increase in the capacity of the blood serum to kill the typhoid bacillus *in vitro*. The second injection usually produces less marked symptoms, but is followed by a further increase of agglutinins in the serum. These observations indicate that the vaccinated person possesses a degree of immunity against the bacillus, a conclusion borne out by the results obtained in the use of the vaccine as a prophylactic against typhoid fever. The earliest observations of the practical value of the method were made in the British army in India, and in the South African War. All the collected statistics showed not only a diminished case incidence among vaccinated persons but also a reduced mortality rate. During the late war the efficacy of vaccine prophylaxis was put to the test on the most extensive scale, with the result that the incidence of enteric fever, an infection which has always been specially prevalent under war conditions, was minimal even in the campaigns waged in tropical and subtropical countries.

In the early stages of the Mediterranean campaign, however, enteric fever was prevalent, but the great majority of the cases were paratyphoid infections, and it is significant that the troops were immunised at that time only against the typhoid bacillus. After the introduction of the combined typhoid-paratyphoid inoculation (*vide* p. 425), enteric infections generally were reduced to negligible proportions. It must be remembered that the duration of the artificial immunity is limited, and if it is necessary to maintain a constant prophylaxis, immunisation should be repeated at yearly intervals. Wright, in his early work on typhoid immunisation, drew attention to the fact that immediately after inoculation there was a fall in the bactericidal power of the blood—the so-called "negative phase"—and considered that this indicated a temporary lowering of resistance. As a result it has often been recommended that vaccination should be carried out some time previous to the exposure to infection. While on general principles it is advisable that the vaccine injections should be given, if possible, before exposure, it is doubtful if a true negative phase in the resistance occurs, and the possibility of such an effect has been overestimated.

Antityphoid Serum.—Chantemesse immunised animals with dead cultures of the typhoid bacillus, and, having found that their sera had protective and curative effects in other animals, used such sera in human cases of typhoid with apparent good result. In the hands of others, however, such a line of treatment has not been equally successful.

The Isolation of the Typhoid Bacillus from Water Supplies is dealt with in the Appendix.

Paratyphoid Fever

In 1898 Gwyn recorded a case clinically resembling typhoid fever, and isolated from the blood an organism then known as the paracolon bacillus, which is now designated *B. paratyphosus*. Since that time numerous outbreaks of intestinal disease, due to this organism, have been observed. During the late war these came into great prominence from the fact that they constituted a prevalent group amongst intestinal infections as a whole. Clinically they may be indistinguishable from true typhoid, and under war conditions exhibited considerable severity, though they frequently take the form of a mild typhoid-like illness which may be transient. The lesions may be like those of typhoid fever with or without ulceration though notably less severe. But the lymphoid tissue may not be

affected in the characteristic way, and the condition is then rather a diffuse catarrhal enteritis sometimes accompanied by gastritis. There may also be peritonitis without perforation, suppuration in, *e.g.*, the spleen, brain, kidney, lymphatic glands, bones, etc. It has been suggested that initially the old term " enteric fever " or " enterica " should be applied to all clinical cases of a typhoid type pending their differentiation into typhoid and paratyphoid fevers by bacteriological methods.

The Paratyphoid Bacilli (*Salmonella Group*).—These organisms have the general characters of the coli-typhoid group, motility being usually active though the flagella are often few in number. They are non-lactose fermenters and produce acid and gas in glucose, mannite, maltose, dulcite, lævulose, galactose, sorbite, and arabinose ; they do not ferment raffinose, saccharose, salicin, or inulin. Of these reactions, that with lactose differentiates them from B. coli, and the production of gas distinguishes them from B. typhosus and B. dysenteriæ. They do not produce indol. Two types occur, denominated respectively " B. paratyphosus A " (*Salmonella paratyphi*) and " B. paratyphosus B " (*Salmonella schottmülleri*), the latter being the commoner in Western Europe though the A type is more prevalent in the East.

Cases of enteric fever have also been reported in the East as being due to a type which is biologically different from the others, and this organism has been designated B. paratyphosus C. It seems to be related serologically to B. suipestifer (*vide* p. 427).

The fermentative capacities of these are identical, but A is the less active—gas formation being often scanty and late in appearance. This is particularly noticeable in regard to dulcite. They present slight differences on ordinary media. On agar, gelatin, and potato, A in its growth rather resembles B. typhosus, while B is more like B. coli ; in litmus milk A produces alkali slowly, while, in the case of B, alkali production is rapid. A difference can also be established in regard to xylose fermentation and the formation of sulphuretted hydrogen in lead acetate agar (*vide* Table, p. 443). In the ultimate differentiation of the two types their reactions with specific agglutinating sera is of essential importance. Colonies of B. paratyphosus B develop a striking character under certain conditions and in this respect differ from colonies of B. paratyphosus A, and B. typhosus. This consists in the formation of an opaque raised margin developing when plates that have been incubated at 37° C. are subsequently allowed to stand at room temperature for twenty-

four to forty-eight hours. This character, which was originally described by Müller, may assist in the recognition of the colonies and the isolation of the organism from mixed growths. Certain strains form rough and smooth colonies on agar plates (Fig. 110), as has been described in the case of B. typhosus (p. 410).

What has been said with regard to pathogenesis, distribution in the body, viability, infection, carriers, epidemiology, and immunity in the case of the typhoid bacillus, is generally applicable also to the paratyphoid bacilli.

As in typhoid fever, patients suffering from paratyphoid develop specific agglutination properties in their blood serum for the causative organism. Thus the serum of a patient infected with B. paratyphosus A may agglutinate this organism,

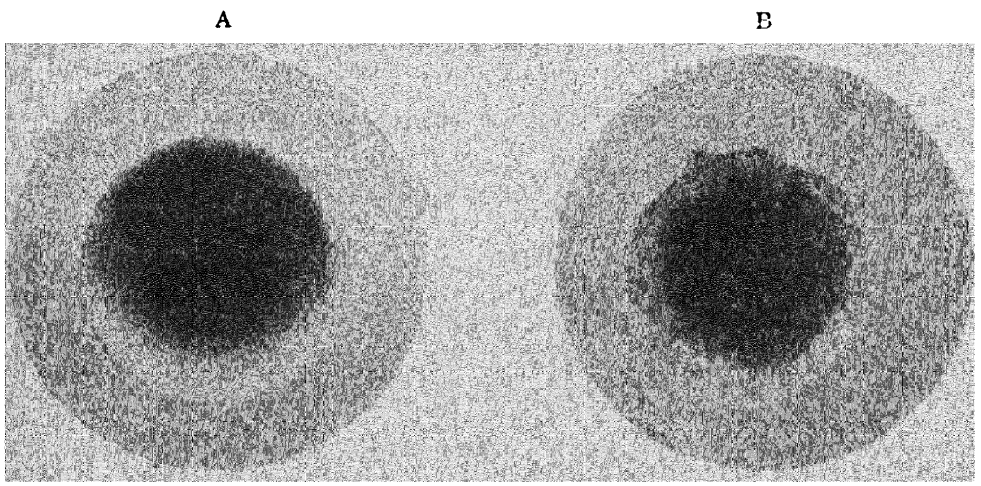


FIG. 110.—A smooth, and B rough, type of colony of B. paratyphosus B. ×40.

but will have little or no effect on B. typhosus or B. paratyphosus B. Co-agglutination effects are slight, and the reaction is usually highly specific.

The principles applicable in the case of B. typhosus to the diagnostic use of the agglutination test also hold for the paratyphoid bacilli. Normal serum effects have to be specially considered in interpreting results, and quantitative tests should always be carried out in which a series of dilutions are tested, preferably by the macroscopic method (*vide* p. 125). Criteria of a positive reaction in terms of the dilution in which agglutination occurs, must also be used. In the case of B. paratyphosus A, marked agglutination in a dilution of 1 : 20 is significant, but with B. paratyphosus B an agglutination result cannot be accepted as diagnostic unless agglutination occurs in a dilution

of 1 : 200. Further, such criteria refer only to persons unvaccinated against these organisms. Repeated tests may often be required to elicit conclusive results, and it is well known that in the paratyphoid A infections the agglutinin development is frequently weak or is almost absent till late in the disease.

Further points regarding the agglutination of these organisms (p. 439) and the methods of isolation (p. 437) will be treated later.

Preventive Inoculation.—All the evidence points to inoculation with the B. typhosus having no effect in protecting against paratyphoid fever. It is therefore now customary, in the prophylaxis of enteric fever, to use for the inoculation a mixture containing in the case of the first dose 500 million B. typhosus, 375 million B. paratyphosus A, and 375 million B. paratyphosus B, and after seven to ten days, as in the original method of typhoid vaccination, a second dose is given, containing double these quantities of the three organisms.

Organisms associated with Food-Poisoning and Allied Bacilli

Organisms biologically related to the paratyphoid bacilli, particularly to B. paratyphosus B, are found associated with cases and outbreaks of acute enteritis following the ingestion of some article of food, frequently meat, contaminated with the particular organism. Such cases were at one time designated "ptomaine poisoning," from the idea originally prevailing that the symptoms were caused by ptomaine substances produced from the bacterial decomposition of the proteins of the food. Such conditions of actual ptomaine poisoning must be exceedingly rare, and the vast majority of cases of food-poisoning are due to the group of bacteria now under consideration, which are all capable of multiplying in the intestine; they may even produce a general blood infection. The condition of botulismus, a special form of food-poisoning, is discussed in a subsequent chapter (p. 540). The meat or food at fault may not, to taste or smell, present any unusual features, but very often there can be isolated from it an organism identical with that derived from the sick individuals. Sometimes it has been proved that the animals from which the meat was derived have been suffering from illnesses probably due to the organisms subsequently found, but this has not always been the case, and meat from healthy animals may be contaminated from extraneous sources. The foods giving rise to poisoning usually belong to the pre-

served-food class, *e.g.* sausages or similar products, but cases also arise from fresh foods, milk, etc.

Bacillus Enteritidis of Gaertner (*Salmonella enteritidis*).—The classical type of such organisms is the B. enteritidis described first by Gaertner (1888) in an outbreak in Saxony of fifty-seven cases of gastro-enteritis following the ingestion of the flesh of a diseased cow. The organism was isolated both from the patients and from the meat. Subsequent similar outbreaks in Germany and in this country were found to be due to the same organism which was demonstrated in the stools and organs of cases. This organism closely resembles B. paratyphosus B in all its general characters, including biochemical reactions (*vide* Table, p. 443), but can be clearly differentiated by agglutination reactions. An antiserum to B. paratyphosus B has little co-agglutinative effect on B. enteritidis, and an antiserum to the latter exhibits little effect on B. paratyphosus. The serum of convalescents may agglutinate the specific organism. The B. enteritidis is very pathogenic for laboratory animals. Often, whatever the channel of infection, there is intense hæmorrhagic enteritis, and usually there is a septicæmia with the occurrence of serous inflammations; the bacilli are recoverable from the solid organs and often from the blood. In man, as the name of the bacillus indicates, the effects are centred in the intestine, where there is usually marked inflammation of the mucous membrane, sometimes attended with hæmorrhage into it; evidence of a septicæmic condition may also exist. Infection may take place by the bacillus itself, and here the illness usually appears within twenty-four hours of the food being partaken of, but symptoms may appear almost at once, in which case they are no doubt due to the action of toxins; here it is important to note that the poisons formed by this type of organism are relatively heat-resisting, so that boiling for a time does not destroy the toxicity.

Since Gaertner's bacillus came to be recognised as a causal agent of "food-poisoning" illnesses of the type described, similar outbreaks in various countries have been carefully investigated bacteriologically, and other members of the paratyphoid (or Salmonella) group have also been described as causal organisms. A great deal of confusion, however, has arisen in their classification and nomenclature. The Salmonella group considered as a whole comprises a number of types all resembling one another in general and biochemical characters but serologically different. Even in serological characters, however, the group relationship among certain types is so close that

agglutinin absorption tests (*vide* p. 440) are necessary for their identification.

The existence of group agglutinins and specific agglutinin in a serum prepared towards a particular bacillus has long been recognised, the former acting on other members of the same group, the latter only on the organism used as antigen. This has been generally explained as being due to the complexity of the antigenic structure of the bacillus in question, some antigens being specific or peculiar to the bacillus, others being possessed in common with other members of the group ; and it has been assumed that the individual bacilli in a pure culture have the same antigenic structure. The work of Andrewes, however, has shown that in the case of the Salmonella group the two kinds of antigen are carried by two different types of bacilli, and his observations have been confirmed by others. In analysing the subject, he first prepared a pure specific serum and a pure group serum. The former was obtained by absorbing the group agglutinin in one antiserum to a particular organism by treatment with an emulsion of another bacillus of the same group ; thus a mono-specific serum resulted. The pure group serum was simply a serum prepared against another bacillus of the group, provided that it was rich in group agglutinin ; presumably it contained no specific agglutinin for the bacillus to be tested. When agar plates were prepared and individual colonies were picked out, it was found that the bacilli of some colonies were agglutinated by the specific serum, practically not at all by the group serum ; whilst the converse held in the case of other colonies. In the mass culture there are accordingly two types of bacilli different in their antigenic structure, one type containing the specific antigen, the other containing a group antigen, though neither is absolutely pure. When, however, subcultures are made from a colony of either type, the other type soon reappears ; that is, the type character is not maintained.

Savage and White in a recent study of the organisms of the Salmonella group associated with food-poisoning have recognised the following types : B. enteritidis (Gaertner), B. aertrycke, B. suipestifer, and certain other varieties designated " Derby," " Stanley," and " Newport."

The B. aertrycke was originally isolated by De Nobele in an outbreak of food-poisoning at Aertrycke, in Belgium. The B. suipestifer or bacillus of hog cholera had previously been described by Salmon and Theobald Smith in swine fever (or hog cholera),which, however, was later shown to be due to a filterable

virus, the B. suipestifer being probably a secondary agent in the disease. The B. aertrycke and B. suipestifer were long regarded as almost identical, and their association with food-poisoning was well recognised.

These organisms have been confused with B. paratyphosus B owing to their close serological relationship with it, and similarly strains designated B. paratyphosus B have been described in food-poisoning cases. Savage and White have now separated these organisms into the different serological types referred to above, and point out that B. paratyphosus B is associated with paratyphoid fever only, and probably never occurs in food-poisoning. B. suipestifer they regard as of very exceptional occurrence in food-poisoning, though B aertrycke is a frequent cause. The so-called "Mutton" type which has been found in a number of outbreaks in this country is probably identical with B. aertrycke. Savage and White regard the B. aertrycke as the commonest cause of Salmonella food-poisoning, while next in frequency is the B. enteritidis. The "Newport" type is also responsible for a certain proportion of cases, but the "Stanley" and "Derby" types are comparatively rare.

In the etiology of food-poisoning the sources of the causative organisms and the mode of contamination of the offending article of food require consideration. B. enteritidis occurs apparently in natural infections in cows, calves, and rats. B. aertrycke is responsible for epizootic enteritis in guinea-pigs, mice, and other rodents. It also produces a similar condition in parrots (psittacosis) and other birds, and is occasionally found in pigs, but not commonly in rats (Savage and White). Thus, in the case of flesh foods, the animal from which the food is derived may have been infected, as in the case of the original B. enteritidis strain As noted above, preserved foods have frequently been responsible for outbreaks, and it has been assumed that growth of the particular organism may have occurred in the material with the formation of toxin which, on ingestion of the food, is responsible for immediate symptoms. As these organisms are prevalent in mice and rats, contamination of food may occur from this source. In certain instances, human carriers of these organisms may be responsible for the food contamination and outbreaks of enteritis following the ingestion of the food.

In the bacteriological diagnosis of Salmonella food-poisoning, cultures should be made from the stools on plates of MacConkey's medium. Single pale colonies are subcultured, and the cultures are investigated first in regard to their biochemical characters and subsequently their serological reactions. Direct agglutination tests with antisera to known B. enteritidis, B. paratyphosus B, and B. aertrycke respectively may be carried out, but careful agglutinin absorption tests may be necessary before the strain can be identified with one of the recognised types. To prove its serological identity, it should be able to absorb from an antiserum to the known type the agglutinins for a known strain of that type. In certain cases, particularly those of a severe nature, the causative organism is present in the blood and can be isolated by blood culture. The sus-

pected article of food, if obtainable, should be carefully examined bacteriologically with a view to isolating the causative organism from it In convalescence, if a bacteriological diagnosis has not previously been made, examination of the patients' serum by direct agglutination tests and agglutinin absorption tests with known food-poisoning strains of different types may elicit proof of the previous infection

The Psittacosis Bacillus.—When parrots are imported from the tropics in large numbers, many may die of a septicæmic condition in which an enteritis, which may be hæmorrhagic, is a marked feature There is intense congestion of all the organs and peritoneal ecchymoses From the spleen, bone marrow, and blood there has been isolated a bacillus having the characters of the paratyphoid or Salmonella group. The parrot is most susceptible to its action, but it also causes a fatal hæmorrhagic septicæmia in guinea-pigs, rabbits, mice, pigeons, and fowls, the bacilli after death being chiefly in the solid organs. From affected parrots the disease appears to be readily communicable to man, chiefly, it is probable, from the feathers being soiled by infective excrement. Several small epidemics have been recognised and investigated in Paris. After about ten days' incubation, headache, fever, and anorexia occur, followed by great restlessness, delirium, vomiting, often diarrhœa, and albuminuria. Frequently broncho-pneumonia supervenes, and a fatal result has followed in about a third of the cases observed. The organism has been isolated from the blood. Savage and White classify this type of organism with B aertrycke.

Danysz's Bacillus and Rat Viruses.—Danysz isolated from an epizootic in field mice an organism of this group, which he introduced for the purpose of killing rats by originating in them through feeding a similar epizootic, and several viruses of this kind are in commercial use for this purpose. These owe any efficiency they possess to the bacillus enteritidis of Gaertner. The efficacy of such agents varies, and the mortality in artificially originated epizootics is from 20 to 50 per cent. Sometimes, apparently under natural conditions, rats develop an immunity to these viruses, and it is doubtful whether they are entirely innocuous to other animals which may partake of the food containing them.

Bacillary Dysentery

Dysentery has long been recognised as including conditions of different etiological and pathological nature. Two main types are now recognised and designated according to the respective causal agent—*amœbic*, due to the *Entamœba histolytica* which is described later (Chapter XXVI.), and *bacillary*, due to a group of organisms designated collectively *B. dysenteriæ*, and including a number of different types. These are related biologically to the coli-typhoid group, and will be considered here.

The first organism of this type to be described is that now known as *B. dysenteriæ Shiga*, observed by Shiga (1898) in cases of bacillary dysentery in Japan. Subsequently workers in different

parts of the world confirmed Shiga's observations (Kruse and others), and similar organisms differing from the Shiga type in certain biological characters were also described (Flexner, Hiss and Russell, and others). It was further recognised that epidemics of dysentery occurring from time to time in lunatic asylums were due to this group, and that the same organisms were responsible for cases of acute enteritis in infants and children—the so-called "summer diarrhœa." Important additions were made to our knowledge of bacillary dysentery during the war, when this condition assumed serious proportions among troops, especially in those campaigns carried on in tropical and sub-tropical areas. The evidence for the relationship of these organisms to the disease consists chiefly in their constant presence in the dejecta in the early stage of the illness and in the agglutination of the associated strains by the serum of patients, but confirmatory evidence has also come from animal experiments and from the therapeutic effects of specific antisera. While different biological types of B. dysenteriæ are recognised, they all present certain common characters and constitute a fairly well-defined group. The differentiation of types depends on variations in fermentation and biochemical reactions and in serological characters.

Bacilli of Dysentery.—The following are the characters common to the group:

Morphological Characters.—The bacilli morphologically resemble the typhoid bacillus, but show more tendency to the cocco-bacillary form, and filamentous forms are comparatively rare. No spore formation occurs. The organisms are non-motile; they are stained readily by the ordinary dyes, and are Gram-negative.

Cultural Characters.—On plates of nutrient agar the colonies resemble those of the typhoid organism, being of smaller size and less opaque than those of the B. coli. In *gelatin* stab culture a whitish line of growth occurs along the puncture, but a superficial film-like growth is usually absent, or at least poorly marked. In plate cultures the superficial growths have often the vine-leaf contour of typhoid colonies. On *MacConkey's medium* the colonies are pale and colourless.

In *peptone bouillon* a uniform haziness is produced. In *litmus milk* there is developed at first a slight degree of acidity, which is followed by a phase of increased alkalinity; no coagulation of the milk ever occurs. On *potato* the organism forms a transparent or whitish layer, which, however, in the course of a few days assumes a brownish red or dirty grey

colour, with some discoloration of the potato at the margin of the growth. As has been indicated, different types behave differently in biochemical and serological reactions. They all ferment glucose without gas production, and with the exception of certain atypical varieties (referred to later) do not ferment lactose. In all fermentation reactions they are non-gas-producing (*vide* Table, p. 443). The classical Shiga type (*Eberthella dysenteriæ*) has no action on saccharose, mannite, maltose, or dulcite, and does not produce indol in peptone water. Another type (*Eberthella paradysenteriæ*), originally described by Flexner, differs from the Shiga variety in the fermentation of mannite and maltose, and in the production of indol. Hiss and Russell described a further type which is similar to the Flexner variety, but differs in the absence of maltose fermentation, and designated it the " Y " type. Thus the dysentery bacilli have come to be differentiated into two main sub-groups—the "non-mannite-fermenters " (Shiga) and the " mannite-fermenters " (Flexner-Y), which latter do not form a homogeneous group either in biochemical reactions or in serological characters. As a further criterion of identity the former react specifically with an agglutinating serum prepared to a known Shiga strain, while the latter are generally agglutinated by an antiserum to the classical Y strain (*vide infra*) or alternatively by a polyvalent antiserum to the Flexner-Y sub-group.

It has been shown that the organisms of the Flexner-Y type represent four different antigenic components designated V, W, X, Z, one of which may predominate in a particular strain so as to give it a separate serological stamp; on this basis Andrewes and Inman classified the group into five main races, namely, V, W, X, Y, and Z, their Y race corresponding generally to the classical Y type. They regarded the Y race as presenting a mixture of the V, W, X, and Z components more evenly balanced than in the other races. Thus an anti-Y serum has a wider agglutination range than sera from other Flexner strains.

Relation to the Disease.—The organism has been found in large numbers in the dejecta, especially in the early stage of the illness, where it may be present in almost pure culture. It does not appear to spread deeply or to invade the general circulation. In the later stages it may be relatively scanty in the stools and difficult to isolate. Apparently the organisms become progressively less numerous during the illness, and may ultimately disappear from the stools, even while the condition is still active. As they diminish, certain other types of intestinal bacteria appear

in considerable numbers in the stool, *e.g.* B. Morgan No. 1 and allied organisms, B. paracolon types (*vide* Table, p. 424), B. fæcalis alkaligenes, B. proteus, fæcal streptococci, etc. (Mackie). When complete recovery occurs, these give place to the usual coliform flora. MacConkey's lactose agar medium is specially suitable for isolation from stools. As the B. dysenteriæ is not a lactose fermenter, the colourless colonies which develop after twenty-four hours are picked out for further investigation.

In the severe and acute cases where death may occur in from one to six days, the chief changes are a marked swelling and corrugation of the mucous membrane of the colon, with hæmorrhage and pseudo-membrane at places. There is extensive coagulation-necrosis with fibrinous exudation and abundance of polymorpho-nuclear leucocytes, and the structure of the mucous membrane, as well as that of the muscularis mucosæ, is often lost in the exudation. Sometimes ulceration occurs; there is also great thickening of the submucosa, with infiltration of leucocytes, these being chiefly of the character of plasma cells. In the more chronic forms the changes correspond, but are more of a proliferative character. The mucous membrane is granular, and superficial areas are devoid of epithelium, whilst ulceration and pseudo-membrane are present in varying degree. In the stools the presence of a large number of markedly degenerated polymorpho-nuclear leucocytes, macrophages and red corpuscles, with the absence of Entamœba histolytica, points to bacillary dysentery; and in this way a tentative diagnosis may be made by simple microscopic examination of the stool pending the fuller bacteriological investigation of the case.

Agglutination.—The serum of patients may agglutinate the particular causal type of dysentery bacillus, and the reaction has been applied in diagnosis. The reaction is often well marked after from six to seven days in the acute cases. In many cases, however, even after a longer interval the serum may fail to react. Agglutination of the Shiga bacillus in a serum dilution of 1 : 50 is usually accepted as being of diagnostic significance. The case of the Flexner-Y type is much more difficult; it is susceptible to agglutination by normal sera to such an extent that probably a positive result with lower dilutions than 1 : 100 cannot be taken as indicating the presence of infection. The serological heterogeneity of the Flexner-Y types (*vide supra*) renders the practical application of the agglutination test difficult for diagnostic purposes, and in routine laboratory diagnosis the isolation of the causative organism constitutes the most conclusive method of bacterio-

logical diagnosis. The application of agglutination tests with immune sera for the identification of strains has been referred to above.

Pathogenic Properties.—Generally it is impossible to produce any effect on laboratory animals by infection *per os.* Shiga, however, obtained characteristic effects by introducing the organism into the stomach of young cats and dogs, and confirmatory results were obtained by Flexner. Such attempts have been specially successful when the virulence of the organism has been previously exalted by intraperitoneal *passage.* The dysentery bacilli when recently isolated exhibit marked pathogenic effects when introduced intravenously in rabbits, a minute amount of culture being sufficient to produce a lethal result. The organisms show marked enterotropism, and inflammatory changes occur in the mucosa of the small and large intestine with frequently excessive hæmorrhages. The bacilli can be recovered from the contents of the intestine, where they may be present in large numbers. These results may also be produced often by subcutaneous injection. In two cases, apparently well authenticated, a dysenteric condition has followed in the human subject from ingestion of pure cultures of the organism.

It has now been well established that the Shiga type of dysentery bacillus produces a diffusible or exo-toxin which can be separated from fluid cultures by filtration. Apparently aerobic conditions are necessary for its production in culture. The results of most observers show that the Flexner-Y strains do not produce a similar diffusible toxin. In this connection it is noteworthy that in general the Shiga strains, as contrasted with the other varieties, are associated with the most severe clinical types of the disease. The toxin of the Shiga type is extremely active in animals, especially rabbits, and, however introduced into the body, may produce a hæmorrhagic enteritis with often a pseudo-membranous exudate on the surface of the mucosa. According to Kanai this toxin in rabbits affects also the central nervous system (medulla and spinal cord), with resulting paralysis, which is a frequent result of inoculation. The toxin is fairly resistant to heat, standing temperatures up to 70° C. without loss of its properties.

An aggressin effect (*vide* p. 165) has also been described in the case of the dysentery bacillus.

The Atypical Dysentery Bacilli.—During the war, bacillary dysentery was extensively investigated, and while the classical types of bacilli formed a large proportion of the strains isolated from

cases, " atypical " varieties were constantly met with. These were frequently found in large numbers in the stools in early cases. Some of them corresponded in all their cultural and biochemical reactions to the Shiga or Flexner-Y types, but failed to react to specific agglutinating sera for these organisms. An organism similar to the Flexner type but inagglutinable by specific antisera was also described by Gettings in an outbreak of asylum dysentery. Certain strains when repeatedly subcultured apparently underwent spontaneous changes in their biochemical characters and displayed fermentative reactions, *e.g.* fermentation of saccharose, dulcite, lactose, which clearly differentiated them from the classical types. Other strains on first investigation showed certain biochemical differences from the classical types, and also reacted negatively with agglutinating antisera. All these types, however, presented characters in common with the recognised dysentery bacilli—being non-motile Gram-negative bacilli, non-gelatin-liquefying, non-gas-producing, fermenting glucose, but varying in the fermentation of saccharose, mannite, dulcite, and lactose, and the production of indol (Mackie). Lactose-fermenting strains otherwise similar to the Flexner type though inagglutinable by antisera were also observed by various workers and regarded as dysentery producers. A saccharose-fermenting type similar otherwise to the Flexner bacillus had also been described by Strong.

The various types met with were carefully studied by Mackie, and proved when recently isolated to be extremely virulent on intravenous injection of rabbits, producing a characteristic hæmorrhagic enteritis similar to that resulting from a similar inoculation with the classical strains. As regards the type of illness due to these organisms, the majority of cases were of the milder type, though severe conditions were not infrequently met with in which atypical organisms were present in large numbers in the early stage.

Sonne Group.—A group of organisms biologically and serologically distinct from the Flexner-Y types has been recognised by Thjøtta and Sonne as dysentery-producing organisms in Norway, Sweden, and Denmark. These organisms are similar to Flexner-Y strains and ferment mannite, but late fermentation of saccharose and lactose occurs. Indol is not formed. Cases due to this type have recently been described by Kerrin and Cruickshank in Scotland.

Schmitz's Bacillus.—This organism is similar to B. dysenteriæ (Shiga), differing, however, in the formation of indol. It has been described in dysentery cases and outbreaks by various observers.

Immunisation.—Both large and small animals have been immunised against the dysentery bacilli and also against toxic filtrates. In the former case the immunisation has been commenced either with non-lethal doses of living cultures, or with cultures killed by heat. The nature of the immunisation is probably complex. When cultures have been used, a bactericidal serum is developed in which immune-bodies and complement (*vide* Chapter VI.) are concerned. When toxin alone is used for immunisation, an antitoxic serum is produced. Accord-

ing to some results, animals immunised with cultures are immune against the toxin, and *vice versa*.

Antisera prepared by immunising large animals against the dysentery bacilli have now been extensively applied in the treatment of acute cases. Polyvalent sera have generally been used. Shiga originally reported favourably regarding the therapeutic value of a polyvalent serum used in Japan in large numbers of cases. The active principles of such sera are both antitoxic and antibacterial. While reports as to their practical value have varied considerably, it may be said that in the severe cases, if sufficiently large doses are given (*e.g.* 50–100 c.c.) and repeated if necessary, favourable results are obtained. In the most acute cases the serum should be given intravenously.

Prophylactic Vaccination.—A difficulty attending the application of vaccines is the extremely toxic effects exerted by killed cultures when injected subcutaneously even in relatively small doses. Shiga obviated this by using a sensitised vaccine (*vide* p. 181), and this method has been followed by others. Vaccine prophylaxis has not, however, been so extensively applied as to allow of conclusions being drawn regarding its practical value.

Infantile Diarrhœa

The etiology of this condition, so prevalent in infants and children in the warmer seasons of the year, has attracted considerable attention from the bacteriological standpoint. It is doubtful whether the condition is to be regarded as due to a single specific agent. Certain of the more severe cases occurring in this country at the present time and in other temperate climates are found on investigation to be due to the dysentery bacilli, mostly of the Flexner-Y type. Such cases occur both sporadically and in groups, and while the stools may present the typical appearances of dysenteric dejecta with blood and mucus present, such appearances may be absent, and the condition would not be designated dysentery in the clinical application of the term.

A careful investigation of the disease in Britain was made some years ago by Morgan, who found no evidence of the association of dysentery bacilli with the condition. In 63 per cent. of the cases investigated, however, he isolated from the stools and intestine a type of organism now generally designated "Morgan's No. 1 bacillus" (*Salmonella morgani*), which is a motile Gram-negative bacillus belonging to the coli-typhoid

group and possessing characteristic fermentative reactions. It produces acid and gas in glucose, but has no effect on lactose, saccharose, mannite, maltose, or dulcite; it produces indol; gelatin is not liquefied. It causes diarrhœa and death in young rabbits, rats, and monkeys, when these animals are fed on cultures. Morgan also found that in diarrhœa cases, the lactose-fermenters, so characteristic of normal fæces, are relatively less numerous and tend to be replaced by non-lactose-fermenting types.

In a study of simple diarrhœal conditions, apart from clinical dysentery, occurring in troops during the Mediterranean campaign, various non-lactose-fermenting Gram-negative bacilli, *e.g.* Morgan's No. 1 bacillus and similar types, " B. paracolon " types (*vide* Table, p. 442), B. fæcalis alkaligenes, B. proteus, etc., were met with in stools and were present often in very large numbers, almost replacing the usual coliform bacilli (Mackie). These organisms were also met with as concomitants of the dysentery bacilli (*vide supra*) in typical dysenteric cases. The question remains unsettled as to whether such organisms are specific pathogenic types which are transmitted from person to person, or whether they are simply intestinal commensals normally scanty, but under certain conditions capable of replacing the usual coliform flora. The evidence collected on this subject is highly suggestive, however, that certain of these organisms are pathogenic entities responsible for diarrhœal conditions not only in young subjects, but also in adults.

Bacillus Fæcalis Alkaligenes (*Alcaligenes fæcalis*) — This organism may conveniently be considered here. It is a motile, Gram-negative, non-sporing bacillus resembling the typhoid bacillus in its morphological and general cultural characters. It is, however, devoid of fermentative properties towards any of the carbohydrate substances used in the testing of the organisms of the colityphoid group (*vide* Table, p. 442). It does not liquefy gelatin. The classical type is actively motile, but reports as to the number and arrangement of its flagella vary. Multiple peritrichous flagella have been described, but strains are met with possessing a single terminal flagellum like that of certain vibrios. Non-motile strains with otherwise the same characters as B. fæcalis alkaligenes may be met with, and it seems probable that organisms classified under this designation represent a number of varieties.

This type of organism may occur occasionally in small numbers in fæces from healthy persons. In certain pathological conditions of the bowel large numbers may be found in the stool, often replacing to some extent the normal B. coli flora, *e.g.* in late cases of bacillary dysentery (*q.v.*) and in non-dysenteric diarrhœa. Blood infections with B. fæcalis alkaligenes have also been described in cases presenting a transient febrile illness.

Isolation and Differentiation of Coli-Typhoid Bacilli by Culture

The existence of a group of intestinal diseases with similar clinical features caused by closely allied bacteria makes the differentiation of these affections and of the causal bacteria a difficult problem. The difficulty is increased where, as in the conditions existing during the recent war, several of these diseases may be simultaneously prevalent, each on a considerable scale. The best solution of the bacteriological problem is found in the isolation of the organisms from the blood or stools of the patient, but here often the best methods may fail to yield cultures, especially when, as has often been the case, the individual does not come under observation till the acute phase of the disease has passed. Moreover, cases occur where more than one pathogenic organism may be present. The isolation method should, however, invariably be attempted.

Post mortem, organisms may be isolated from the intestine, particularly from ulcerative lesions if such occur, as in enteric fever and in dysentery; cultures may be obtained from the gall-bladder, and here the causative organism may occur in a state of purity as in enteric fever; the particular organism may also be isolated from the spleen, mesenteric glands, and even the heart blood, as in enteric fever and in bacterial food-poisoning.

During life, the bacilli may be obtained in culture in the following ways:

(*a*) *From the Blood.*—The bacilli of the group may be isolated from the blood by ordinary methods (see p. 145), but a special method is often also used. In this, 5 c.c. of the blood are placed in 10 c.c. sterilised ox bile, or in 50 c.c. sterilised 0·5 per cent. solution of sodium taurocholate; the mixture is incubated for from one to seven days, and from time to time the presence of non-lactose fermenters is tested by inoculating MacConkey plates. In typhoid and paratyphoid infections the organisms have been stated to have been isolated from the blood during the prefebrile stage and are very usually present during the first seven to ten days of the illness and in relapses. In bacillary dysentery a blood infection is not common. It may be said generally that the isolation of an organism of the group from the blood during an acute illness probably furnishes the most significant evidence as to its being the cause of the condition present.

(*b*) *From the Stools.*—In the case of a solid or semisolid stool, a dense emulsion is prepared in saline and allowed to stand in a tube till the solid particles have settled; one or two loopfuls from the supernatant fluid are then used for inoculating plates of MacConkey's medium by the methods described on p. 74—the plate being incubated with the medium uppermost. The brilliant green enrich-

ment method may be recommended for the typhoid-paratyphoid group (p. 59). In any case the presence on MacConkey plates of colourless colonies of Gram-negative bacilli constitutes presumptive evidence of the existence of pathogenic members of the coli-typhoid group in the fæces. Some of these colonies should now be picked off into peptone-water and into mannite tubes. The former are used after a few hours' incubation for investigating motility—the latter for observing fermentation. Preliminary indications of the type of organism may be obtained as follows : growth in mannite without acid or gas suggests the B. dysenteriæ Shiga, if the organism is non-motile, or Morgan's No. 1 bacillus, if motile ; the development of acid without gas may be due to the B. typhosus, if the bacillus be motile, or to the B. dysenteriæ (Flexner-Y), if non-motile ; a culture showing acid and gas associated with motility in the organism indicates one of the paratyphoid bacilli, B. Gaertner, etc. Agglutination observations may now be made (*vide infra*), and a set of fermentation and biochemical tests appropriate to the organism suspected to be present may be put up ; it is well to include amongst these a gelatin tube, to exclude proteus types, and one of lactose—the latter to be kept under observation for some days in case the bacillus be a slowly lactose-fermenting B. coli. In this connection the fact must always be borne in mind, in dealing with any coli-typhoid bacillus, that its fermentative capacities may be only slowly manifested.

In any extensive investigation of intestinal infections, atypical bacilli will from time to time be encountered which show variations from the characters of the classical type.

The fæces always constitutes an important source of cultures in the diseases under consideration. In typhoid infections the organisms tend to be most numerous in the stools in the third week. B. paratyphosus A is most numerous about the twelfth day and B. paratyphosus B at the end of the second week. It must be remembered, however, that compared with the number of B. coli in the stool the pathogenic organisms may be relatively scanty and technically difficult to isolate. For this reason the brilliant green selective enrichment method has proved of great value in the isolation of the typhoid-paratyphoid group.

In bacillary dysentery, the organisms are usually very numerous during the first few days of the illness but gradually disappear from the stools and are replaced by various "concomitant" organisms (p. 432). Certain of these are non-lactose fermenters, *e.g.* B. Morgan No. 1, B. paracolon types, etc.

(*c*) *From the Urine.*—In typhoid fever the bacilli are present in at least 25 per cent. of cases, especially late in the disease, probably chiefly where there are groups of the organisms in the kidney substance. The organism can also be found in paratyphoid infections. For methods of examining the urine, see pp. 149, 401. In these conditions bacilluria may occur in which the typhoid or paratyphoid bacilli are so numerous in the urine as to render it turbid.

The ultimate differentiation of the pathogenic varieties of the coli-typhoid group is effected by the study of their agglutination reactions with sera prepared by immunising animals with known

typical strains of the particular organism (*vide infra*). For identification, the suspected strain should agglutinate to approximately the "end-titre" of the serum, and tests should be carried out by the quantitative method (*vide* p. 125). Agglutinin-absorption tests may be required for the detailed identification of types of food-poisoning organisms.

General Considerations regarding the Agglutination Reactions of the Coli-Typhoid Bacilli and their Relation to the Identification of Strains

We have seen that all the members of the group may produce agglutinins in the serum during the infection they originate. The specificity of the reaction may be sufficient in certain cases to constitute a basis on which a diagnosis of the condition can be based. Thus the strain actually isolated from a patient is agglutinated frequently by his serum. The reaction is in practice elicited by the use of standard strains isolated from previous cases.

The diagnosis of the nature of an intestinal infection by the agglutination method is relatively simple if the serum agglutinates only one species of bacterium. The chief point to be borne in mind here is that sometimes the serum of a *normal* individual may agglutinate certain organisms. Such a phenomenon, however, is usually only met with when the serum is in relatively high concentration. The criteria regarding the dilutions with which agglutination must occur in order that the result may be considered positive are given on pp. 420, 424, 432.

Difficulty in interpreting observations of agglutination reactions is met with when the serum agglutinates more than one member of the group. From this point of view the pathogenic members of the coli-typhoid group fall into two sub-groups, one of which contains the typhoid bacillus, the paratyphoid bacilli and allied organisms, and the other, the dysentery bacilli; a serum which agglutinates frankly a member of one sub-group usually has little or no effect on the members of the other sub-group, but may agglutinate several members of its own group. The occurrence of such cross-agglutination is attributed to the fact that the causal organism in an infection not only stimulates the production of agglutinins towards itself (primary or homologous agglutinins), but also of agglutinins acting on kindred species (secondary, heterologous or group agglutinins, or co-agglutinins). The primary agglutinins are usually formed in much greater amount than the secondary; and careful quantitative

tests usually elicit this difference. Among certain organisms group agglutination may be so marked as to render direct agglutination tests insufficient for differentiation. This will be referred to later. A serum may, however, contain primary agglutinins to more than one organism. This may arise where more than one infection exists at the same time, or where primary agglutinins originating from a previous infection persist in the body, or where such persistence is the relic of a previous preventive inoculation, *e.g.* with typhoid-paratyphoid vaccine. It is an interesting fact that a paratyphoid infection seems to have the effect of stimulating a fresh formation of primary agglutinins against the B. typhosus if these be already present in the serum following typhoid inoculation.

In order to surmount the difficulties arising from such complications some observers have used the method of making frequent—it may be daily—estimations during an illness of the highest dilutions in which the serum will agglutinate each of the organisms which may be suspected to be the causal agent. This method has been specially elaborated by Dreyer, Ainley Walker and Gibson, who hold that the study of the curves of the agglutinin content of the serum gives valuable information. Thus, a regular and marked rise in the curve of one of the typhoid-paratyphoid sub-group, to a maximum between the sixteenth and twenty-fourth day (especially between the eighteenth and twentieth), with a gradual fall thereafter indicates an infection with that bacillus; if in such a case primary agglutinins are present towards other members of the sub-group (due, it may be, to a previous vaccination), the curves of these residuary agglutinins either show no change or a slight rise with a fall to their initial levels, or a marked rise, synchronous with or slightly earlier than that of the curve for the infecting organism.

In trying to differentiate between primary and secondary agglutinins the absorption method (p. 126) may also be used. Castellani found that, when an animal had been immunised with the B. typhosus, this organism would *in vitro* remove from the serum not only the primary typhoid agglutinins, but also the secondary agglutinins, which might act on allied organisms, whereas *in vitro* treatment ("absorption") with a heterologous organism removes only the secondary agglutinin for that organism without influencing the primary agglutinin. Castellani therefore put forward the view that by this means primary could be differentiated from secondary agglutinins, and consequently pure infections could be distinguished from mixed. The method has been applied in human infections and may provide data of value, especially when these are correlated with the results of the other methods described.

From what has been said it will be readily gathered that agglutination reactions are of great value in the diagnosis of intestinal infections, and may enable a positive opinion to be given in cases where attempts to isolate the causal organism by culture fail. This is especially true of infections which have reached a late stage in which it may be impossible to cultivate the causal organism.

Identification of Unknown Strains by Agglutination Reactions.—If the relation of a bacillus to its antiserum is specific, it is obvious that the properties of such a serum can be utilised for the recognition of bacilli of unknown species. Thus if the serum of an animal immunised with B. typhosus agglutinates in high dilution a bacillus with the cultural characters of this organism, the unknown strain is almost certainly the typhoid bacillus ; this is especially likely to be the case if the two organisms are clumped by approximately the same dilution of the serum. On this principle is now based the ultimate determination of the species to which a culture belongs. It can be applied immediately after the organism has been classified into its cultural group by its behaviour towards mannite and by the presence or absence of motility (*vide* p. 438). In the use of the test, high-titre sera obtained by the immunisation of animals with historic or otherwise reliable strains are employed. In routine work antisera for the following organisms are required : B. typhosus, B. paratyphosus A, B. paratyphosus B, B. of Gaertner, B. aertrycke, B. dysenteriæ Shiga, and B. dysenteriæ Y (Hiss and Russell strain). The anti-Y serum is used in the identification of the Flexner-Y types (*vide* p. 431), but a polyvalent Flexner-Y antiserum may be substituted. The titre of each serum to the strain which produced it must be known. In the identification of bacterial strains the macroscopic technique described in Chapter IV. should be employed. In the differentiation of types belonging to the salmonella group, absorption tests are necessary (*vide* p. 428), and for identification the unknown strain should be capable of absorbing from the serum the agglutinins for the homologous organism.

In any extended investigation of intestinal infections, bacilli may be met with similar in biochemical and other cultural tests to specific pathogenic types though inagglutinable by antisera to the species they resemble. Certain of these are found on repeated subculture to agglutinate ultimately with the appropriate antisera and can thus be identified with a recognised type. A strain, therefore, which on first isolation conforms to a specific type but fails to react with the respective antiserum should be

TABLE SHOWING CHARACTERS OF THE BACILLI OF THE COLI-TYPHOID GROUP

	Motility.	Gelatin.	Glucose.	Lactose.	Saccharose.	Mannite.	Dulcite.	Adonite.	Inulin.	Inosite.	Milk.	Indol.	Voges and Proskauer Reaction.	Xylose.	Blackening of Lead Acetate Agar.
B. coli communis (Escherich)	+	–	A.G.	A.G.	–	A.G.	A.G	–	–	–	A.C.	+	–		
B. MacConkey No. 71 ("B. coli communior")	+	–	A.G.	A.G.	A.G.	A.G.	A.G.	–	–	–	A.C.	+	–		
B. acidi lactici (Hüppe) (B. coli group)	–	–	A.G.	A G	–	A.G.	–	A.G.	–	–	A.C.	+	+		
B. lactis aerogenes (B. coli group)	–	–	A.G.	A G.	A.G.	A G.	–	A.G.	–	A.G.	A.C.	–	–		
B. Friedländer[1]	–	–	A.G.	A G. or A.	A.G.	A G.	A.G.	A.G.	–	A.G.	A.C.	+	–		
B. vesiculosus (B. coli group)	–	–	A.G.	A.G.	–	A.G.	–	–	–	–	A.C.	+	–		
B. coli anaerogenes type	+	–	A.	A.	A.	A	A.	–	–	–	A.	+	–		
B. cloacæ[1]	+	+	A.G.	A G.	A.G.	A.G	–	–	–	A.	A.C.	+	+		
B. "paracolon" types[2]	+ or –	–	A.G.	–	–	A.G	– or A.G.					+ or –			
B. Morgan No. 1	+	–	A.G.	–	–	–	–				Alk. (produced slowly)	–	–		
B. fæcalis alkaligenes	+	–	–	–	–	–	–					–	–		
B. typhosus	+	–	A	–	–	A.	– or A.					–	+		

B. paratyphosus A.	+	–	A.G.	–	–	A.G.	A.G.			Alk. (produced slowly)	–	–	–
B. paratyphosus B. . . . B. enteritidis (Gaertner) . . B. aertrycke	+	–	A G.	–	–	A.G.	A.G.			Alk. (produced rapidly)	–	A.G.	+
B. suipestifer	+	–	A G.	–	–	A.G.	– A. or A.G slow.			ditto.	–	A.G.	–
B. dysenteriæ (Shiga) . . .	–	–	A.	–	–	–	–			Alk. (produced slowly)	–		
B. dysenteriæ (Flexner-Y types)[3] .	–	–	A.	–	–	A.	–			Alk. (produced slowly)	– + or –		

+ In Motility column = presence of motility; in Gelatin = liquefaction; in Indol = presence of indol: in Voges and Proskauer = presence of Reaction. – In Motility column = absence of motility; in Gelatin = no liquefaction; in Indol = absence of indol; in Voges and Proskauer = absence of reaction; in other columns = absence of change 'A.' = Acid production; "G." = Gas; "C." = Clot; "Alk." = development of alkalinity.

[1] MacConkey, *Journal of Hygiene*, v, 333; ix, 86.

[2] Mackie, *Journal of Hygiene*, 1919, xviii. 69

[3] From *Maltose* acid (but no gas) may be formed or there may be no change.

NOTE.—In general, only those characters are given which are important in identification and differentiation.

retested after several subcultures on artificial medium. True strains of B. typhosus, B. paratyphosus, and B. dysenteriæ may be found to behave in this fashion. On the other hand, such inagglutinable strains may remain inagglutinable and are to be differentiated from the specific organisms they resemble, as, *e.g.*, B. "paracolon" types (*vide* pp. 405 and 442), atypical dysentery bacilli, etc.

CHAPTER XVI

THE VIBRIO CHOLERÆ AND ALLIED ORGANISMS

Introductory.—Previously to 1883 practically nothing of value was known regarding the nature of the virus of cholera. In that year Koch discovered the organism now generally known as the "comma bacillus" or the *Vibrio choleræ*. He obtained pure cultures of the organism from a large number of cases of cholera, and described their characters. The results of his researches were given at the first Cholera Conference at Berlin in 1884.

In considering the bacteriology of cholera, it is to be borne in mind that in this disease, in addition to the evidence of great intestinal irritation, accompanied by profuse watery discharge, and often by vomiting, there are also symptoms of general systemic disturbance which cannot be accounted for merely by the withdrawal of water and certain substances from the system. Such symptoms include the profound general prostration, cramps in the muscles, extreme cardiac depression, the cold and clammy condition of the surface, the subnormal temperature, suppression of urine, etc. These, taken in their entirety, are indications of a general poisoning in which the circulatory and thermo-regulatory mechanisms are specially involved. In some, though rare, cases, known as *cholera sicca*, general collapse occurs with remarkable suddenness, and is rapidly followed by a fatal result, whilst there is little or no evacuation from the bowel, though *post mortem* the intestine is distended with fluid contents. As the characteristic organisms in cholera are present mainly in the intestine, the general disturbances are to be regarded as the result of toxic substances absorbed from the bowel. It is also to be noted that cholera is a disease of which the onset and course are much more rapid than is the case in most infective diseases, such as typhoid and diphtheria; and also that recovery, when it takes place, does so more quickly. The two factors to be correlated with these facts are: (*a*) a rapid multiplication of organisms, (*b*) the production of rapidly acting toxins.

The Cholera Vibrio (*Vibrio comma*).—*Microscopical Char-*

acters.—The cholera vibrios, as found in the intestines in cholera, are small organisms measuring about 1·5 to 2 μ in length, and rather less than 0·5 in thickness. They are distinctly curved in one direction, hence the appearance of a comma (Fig. 111); most occur singly, but some are attached in pairs and curved in opposite directions, so that an S-form results. Longer forms are rarely seen in the intestine, but in cultures in fluids, as may be well seen in hanging-drop preparations, they may grow into spiral filaments, showing a number of turns. Different strains may show considerable variations in their microscopic appearances. In film preparations made from the intestinal contents in typical cases, these organisms may be present in enormous numbers and in almost pure culture.

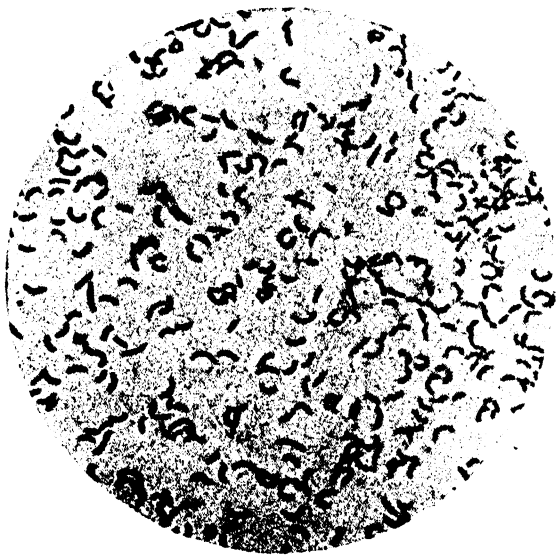


FIG. 111.—Cholera vibrios, from a culture on agar of twenty-four hours' growth. Stained with weak carbol-fuchsin. ×1000.

They possess very active motility, which is most marked in the single forms, and this is due to a single terminal flagellum (Fig. 112). It is very delicate, and measures four or five times the length of the organism. Cholera vibrios do not form spores. In old cultures the organisms may present great variety in size and shape. Some are irregularly twisted filaments, some-



FIG. 112.—Cholera vibrios stained to show the terminal flagella. See also Plate IV., Fig. 19. ×1000.

globose, some clubbed at their extremities, and also showing irregular swellings along their course; others are short and thick, and may have the appearance of large cocci, often staining faintly. All these changes in appearance are to be classed together as *involution forms* (Fig. 113).



FIG. 113.—Cholera vibrios from an old agar culture, showing irregularities in size and shape, with numerous faintly-stained coccoid bodies—involution forms. Stained with fuchsin. ×1000.

Staining.—Cholera vibrios stain readily with the usual basic aniline stains, though Löffler's methylene-blue or weak carbol-fuchsin is specially suitable. They are Gram-negative.

Distribution within the Body.—The chief fact in this connection is that the vibrios are practically confined to the intestine. Certain observations show that they may be found sometimes in the internal organs, and especially in the gall-bladder and biliary passages. Greig found, in a large series of post-mortem examinations, that the cholera organism was present in the gall-bladder in more than a quarter of the cases, and that, in a considerable number of these, distinct pathological changes were present. He found it also in the urine, lungs, and spleen. Another interesting fact observed by him was that in rabbits inoculated intravenously with the living organism for the purpose of obtaining agglutinating sera, infection of the gall-bladder and the formation of gall-stones not infrequently occurred. The all-important factor in the pathology of the disease, however, is the absorption of toxins from the bowel. In cases in which there is the characteristic "rice-water" fluid, the lower half of the small intestine is the part most affected. Its surface epithelium becomes shed in great part, and the flakes floating in the fluid consist chiefly of masses of epithelial cells and mucus, amongst which are numerous vibrios. The vibrios also penetrate the follicles of Lieberkühn, and may be seen lying between the basement membrane and the epithelial

lining, which becomes loosened by their action. In some very acute cases there may be relatively little desquamation of epithelium, the intestinal contents being a comparatively clear fluid containing the vibrios in large numbers. In other cases of a more chronic type, the intestine may show more extensive necrosis of the mucosa and a considerable amount of hæmorrhage into its substance, along with formation of false membrane at places. The intestinal contents in such cases are blood-stained and foul-smelling, there being a great proportion of other organisms present besides the cholera vibrios.

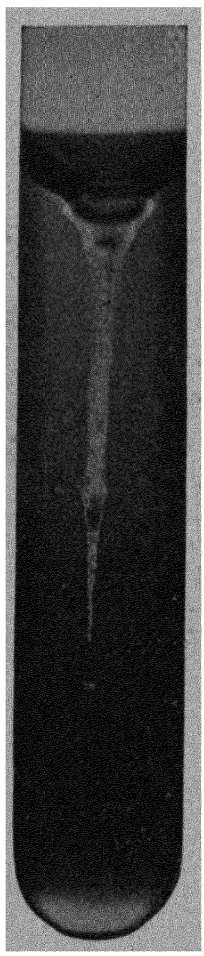


FIG. 114. — Puncture culture of the cholera vibrio in peptone gelatin — six days' growth. Natural size.

Cultivation.—(For methods of isolation, see p. 459.)

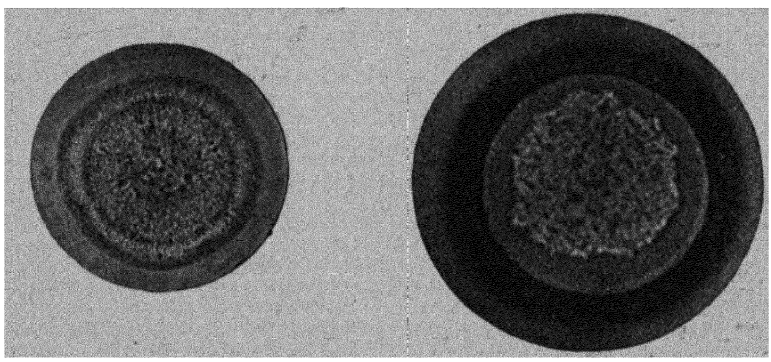
The cholera vibrio grows readily on all the ordinary media, and, except on potato, growth takes place at the ordinary room temperature. The most suitable temperature, however, is 37° C., and growth usually stops about 16° C., though in some cases it has been obtained at a lower temperature. Abundant growth occurs on media with sufficiently alkaline reaction to inhibit the growth of many intestinal bacteria, *e.g.* Dieudonné's medium, p. 56.

On *agar*, the colonies after twenty-four hours' incubation are greyish-white transparent discs, corresponding in size to colonies of the coli-typhoid group, with a well-defined circular outline. A stroke culture gives a uniform growth of similar appearance. Older growths develop a brownish-yellow tint.

Peptone Gelatin.—On this medium the organism grows well and produces liquefaction. In puncture cultivations at 22° C. a whitish line appears along the needle track, at the upper part of which liquefaction commences, and as evaporation quickly occurs, a small bell-shaped depression forms, which gives the appearance of an air-bubble. On the fourth or fifth day the following appearance may be seen. There is at the surface the bubble-shaped depression; below this there is a funnel-shaped area of liquefaction, the fluid being only slightly turbid, but

showing at its lower end thick masses of growth of a more or less spiral shape in the thin line of liquefaction (Fig. 114). (It is to be noted that considerable variations in the degree and rate of liquefaction of gelatin are observed.) At a later stage liquefaction spreads and may reach the wall of the tube. When the organism is subcultured over a long period of time, it may lose to a large extent the property of liquefying gelatin.

On *gelatin plates* the colonies are somewhat characteristic. They appear as minute whitish points, visible in twenty-four to forty-eight hours, the surface of which, under a low power of the microscope, is irregularly granular or furrowed (Fig. 115, A); but considerable variations in the appearances are met with. Liquefaction occurs, and the colony sinks into the small cup formed, the plate then showing small sharply marked rings



A. B.

FIG. 115.—Colonies of the cholera vibrio on a gelatin plate—three days' growth. A shows the granular surface, liquefaction just commencing; in B liquefaction is well marked.

around the colonies. Under the microscope the outer margin of the cup is circular and sharply marked. Within the cup the liquefied portion forms a ring which has a more or less granular appearance, whilst the mass of growth in the centre is irregular and often broken up at its margins (Fig. 115, B).

Growing on *solidified blood serum* the cholera vibrio slowly liquefies the medium. On *potato* at a temperature of from 30° to 37° C. a moist layer appears, which assumes a colour varying from yellowish-brown to pink.

In *bouillon* with alkaline reaction the organism grows very readily, a general turbidity resulting in twelve hours at 37° C. while the surface shows a well-marked pellicle composed of vibrios in a very actively motile condition. Growth takes place under the same conditions equally rapidly in peptone solution (1 per cent. with 0·5 per cent. sodium chloride added).

Vibrio choleræ produces acid, without gas formation, from glucose, saccharose, mannite, and maltose; late fermentation of lactose, with acid production, may occur, namely, after two to three days, but different strains show variation in the power of fermenting this sugar.

In *milk* also the organism grows well, and produces no coagulation nor any change in its appearance, at least for several days.

On all the media the growth of the cholera vibrio is relatively rapid, and especially is this the case in peptone solution and in bouillon, a circumstance of importance in relation to its separation in cases of cholera (*vide* p. 459).

The cholera organism is one which grows much more rapidly in the presence of oxygen than in anaerobic conditions; under strict anaerobiosis very little growth occurs.

Cholera-Red Reaction.—This is a constant reaction, and though it is not peculiar to the cholera vibrio the number of organisms which give the reaction under the conditions mentioned are comparatively few. The test is made by adding a few drops of pure sulphuric acid to a culture in peptone solution (1 per cent.) which has been incubated for twenty-four hours at 37° C.; in the case of the cholera vibrio a reddish-pink colour is produced. This is due to the fact that both indol and nitrite are formed, and hence, in applying the nitroso-indol test, the addition of a nitrite is not necessary (*vide* p. 66). It is essential that the sulphuric acid should be pure, for if traces of nitrites are present the reaction may be given by an organism which has not the power of forming nitrites.

Hæmolytic Test.—The classical V choleræ has usually been described as non-hæmolytic, whereas other vibrios likely to be confused with it are strongly hæmolytic when growing on blood agar, and produce well-marked areolæ of laking round the colonies. Strains of vibrios may be met with, having the serological characters of V. choleræ, which are definitely hæmolytic, *e.g.* the so-called El Tor vibrio (*vide* p 457). Further, strains which appear to be non-hæmolytic after twenty-four hours' growth on blood agar may later produce a clear zone round each colony (Greig). It has, however, been found by several observers that the hæmolytic test is best carried out with a fluid culture. Greig, for example, adds varying amounts, up to 1 c.c. of a three days' culture in alkaline broth to 1 c.c. of a 5 per cent. suspension of goat's corpuscles, the whole being made up to 2 c.c, and thoroughly mixed. The tubes are placed in the incubator for two hours at 37° C., and then in the ice-chest overnight, the results being read next day. He found after testing more than 300 strains of true cholera vibrios that none of them produced hæmolysis, whereas this results with organisms of the El Tor type referred to. In view of the fact that

among strains serologically identical variation in hæmolytic properties occurs, it is doubtful whether special stress should be laid on the presence or absence of this character in the identification of V. choleræ.

Viability and Infection.—In its resistance against heat, the cholera vibrio corresponds with most spore-free organisms, and is killed in an hour by a temperature of 55° C., and much more rapidly at higher temperatures. It has comparatively high powers of resistance against great cold, and has been found alive after being exposed for several hours to the temperature of −10° C. It is, however, killed by being kept in ice for a few days. Against the ordinary antiseptics it has comparatively low powers of resistance, and Pfuhl found that the addition of lime, in the proportion of 1 per cent., to water containing the cholera organisms was sufficient to kill them in the course of an hour.

As regards the powers of resistance in ordinary conditions, the following facts may be stated. In cholera stools kept at the ordinary room temperature, the cholera organisms are rapidly outgrown by putrefactive bacteria, but in exceptional cases they have been found alive even after two or three months. In most experiments, however, attempts to cultivate them even after a much shorter time have failed. The general conclusion may be drawn from the work of various observers, that the vibrios do not multiply freely in ordinary sewage water, although they may remain alive for a considerable period of time. On moist linen, as Koch showed, they can flourish rapidly. Though we can state generally that the conditions favourable for the growth of the cholera vibrio are a warm temperature, moisture, a good supply of oxygen, and a considerable proportion of organic material, we do not know the exact circumstances under which it can flourish for an indefinite period of time as a saprophyte. The fact that the area in which cholera is an endemic disease is so restricted, tends to show that the conditions for a prolonged growth of the vibrio outside the body are not usually supplied. During recent epidemics the cholera organism has been cultivated from the stools of a considerable number of people suffering from slight intestinal disturbance, and even from the stools of quite healthy individuals; these may be regarded as "cholera carriers." Numerous observations, carried out both on convalescents and on contacts having the vibrio in the stools, show that in the great majority of cases it dies out after two or three weeks and usually earlier; it has, however, been found as long as twelve months afterwards. Greig has found that

the excretion of the organism in the stools of carriers is of an intermittent character; accordingly several examinations are necessary before they can be pronounced free. As in the typhoid carrier, the organisms apparently persist in the gall-bladder. There is no doubt that carriers play an important part in the spread of the disease, and can originate epidemics.

Cholera organisms are, as a rule, rapidly killed by being thoroughly dried, and it is inferred from this that they cannot be carried in the living condition for any great distance through the air, a conclusion which is well supported by observations on the spread of the disease. Cholera is commonly transmitted by means of water or food contaminated by the organism, and there is no doubt that contamination of water supplies by choleraic discharges is the chief means by which communities are rapidly infected. It has been shown that if flies are fed on material containing cholera vibrios, the organisms may be found alive within their bodies twenty-four hours afterwards. And further, Haffkine found that sterilised milk might become contaminated with cholera organisms if kept in open jars to which flies had free access, in a locality infected by cholera. Thus infection may be carried also by this agency.

Experimental Inoculation.—In considering the effects of inoculation with the cholera organism, we are met with the difficulty that none of the lower animals, so far as is known, suffers from the disease under natural conditions. Accordingly, attempts to induce the multiplication of the organism within the intestine of animals, by artificially arranging favouring conditions, occupied a prominent place in the early experimental work. We shall give a short account of such experiments:

Nikati and Rietsch were the first to inject the organisms directly into the duodenum of dogs and rabbits, and they succeeded in producing, in a considerable proportion of the animals, a choleraic condition of the intestine. These experiments were confirmed by other observers, including Koch. Thinking that probably the vibrio, when introduced by the mouth, is destroyed by the action of the hydrochloric acid of the gastric secretion, Koch first neutralised this acidity by administering to guinea-pigs 5 c.c of a 5 per cent. solution of carbonate of soda, and some time afterwards introduced a pure culture into the stomach by means of a tube. As this method failed to give positive results, he tried the effect of artificially interfering with the intestinal peristalsis by injecting tincture of opium into the peritoneum (1 c c. per 200 grams weight), in addition to neutralising as before with the carbonate of sodium solution. The result was remarkable, as thirty out of thirty-five animals treated died with symptoms of general prostration and collapse. Death occurred after a few hours. *Post mortem* the small intestine was distended, its mucous membrane congested, and it contained a

colourless fluid with small flocculi and the cholera organisms in practically pure cultures. Koch, however, found that when the vibrios of Finkler and Prior, of Deneke (*vide infra*), and of Miller, were employed by the same method, a certain, though much smaller, proportion of the animals died from an intestinal infection. Though the changes in these cases were not so characteristic, they were sufficient to prevent the results obtained with the cholera organism from being used as a demonstration of the specific relation of the latter to the disease.

Some additional facts with regard to choleraic infection of animals may be mentioned. For example, Zabolotny found that in the marmot an intestinal infection readily takes place by simple feeding with the organism, there resulting the usual intestinal changes, sometimes with hæmorrhagic peritonitis—the organisms, however, being present also in the blood. And of special interest is the fact, discovered by Metchnikoff, that in the case of young rabbits shortly after birth a large proportion die of choleraic infection when the organisms are simply introduced along with the milk, as may be done by infecting the teats of the mother. Further, from these animals thus infected the disease may be transmitted to others by a natural mode of infection. In this affection of young rabbits many of the symptoms of cholera are present. Many of these experiments were performed with the vibrio of Massowah, which is now admitted not to be identical with the classical cholera organism, others with a cholera vibrio obtained from the water of the Seine

Experiments performed by direct inoculation also supply interesting facts.

Intraperitoneal injection in guinea-pigs is followed by general symptoms of illness, the most prominent being distension of the abdomen, subnormal temperature, and, ultimately, profound collapse. There is peritoneal effusion, which may be comparatively clear, or may be somewhat turbid and contain flakes of lymph, according to the stage at which death takes place. If the dose is large, organisms are found in considerable numbers in the blood and also in the small intestine, but with smaller doses they are practically confined to the peritoneum. Kolle found that when the minimum lethal dose was used in guinea-pigs, the peritoneum might be free from living organisms at the time of death, the fatal result having taken place from an intoxication (cf. Diphtheria, p. 472). These and other experiments show that though the organisms undergo a certain amount of multiplication when introduced by the channels mentioned, still the tendency to invade the tissues is not a marked one. On the other hand, the symptoms of general intoxication are always pronounced.

The cholera vibrio is markedly "enterotropic," and by the intravenous injection in rabbits of cultures, a pathological picture resembling that in human cholera can be produced (Mackie). The animals die in twenty-four to forty-eight hours, and before death there is marked diarrhœa; at autopsy the small intestine is markedly distended with a milky mucous fluid containing whitish flakes of desquamated epithelium and resembling the "rice-water" stool of cholera. Large numbers of vibrios are present in the intestine, and the gall-bladder is also heavily infected. It must be noted, however, that other vibrios produce a similar effect, *e.g.* the so-called paracholera vibrios.

It will be seen from the above account that the evidence obtained from experiments on animals is on the whole indicative of the specific pathological relationships of the organism, especially when it is borne in mind that animals do not in natural conditions suffer from the disease.

Experiments on the Human Subject.—Experiments have also been performed in the case of the human subject, both intentionally and accidentally. In the course of Koch's earlier work, one of the workers in his laboratory was seized with severe choleraic symptoms. The stools were found to contain cholera vibrios in enormous numbers. Recovery, however, took place. In this case there was no other possible source of infection than the cultures with which the man had been working, as no cholera was present in Germany at the time. A considerable number of experiments have been performed on the human subject, which certainly show that in some cases more or less severe choleraic symptoms may follow ingestion of pure cultures, whilst in others no effects may result. The former was the case, for example, with Emmerich and Pettenkofer, who made experiments on themselves, the former especially becoming seriously ill. In the case of both, diarrhœa was well marked, and numerous cholera vibrios were present in the stools, though toxic symptoms were proportionately little pronounced. Metchnikoff also, by experiments on himself and others, obtained results which convinced him of the specific relation of the cholera vibrio to the disease. Lastly, we may mention the case of Dr. Örgel in Hamburg, who contracted the disease in the course of experiments with the cholera and other vibrios, and died in spite of treatment. This took place at a time when there was no cholera in Germany. As the result also of observations on cholera epidemics and of cholera carriers, it may be concluded that only a certain proportion of people are extremely susceptible to cholera, and the facts just mentioned are of the greatest importance in establishing the relation of the organism to the disease.

Toxins.—The general statement may be made that filtered cholera cultures as a rule have little toxic action—that is, comparatively little exotoxin is produced by the organism. It was, however, shown by R. Pfeiffer that the dead organisms were highly toxic, and that they produced, on injection into guinea-pigs, the same phenomena as living cultures, profound collapse with subnormal temperature being a prominent feature. Pfeiffer considered that the toxic substances are contained in the bodies of the organisms,—that is, they are *endotoxins*,—and

that they are only set free by the disintegration of the latter. He showed also that when an animal is inoculated intraperitoneally with the cholera organism, and then some time later anti-cholera serum which produces bacteriolysis is injected, rapid collapse with a fatal result may ensue, apparently due to the liberation of the endotoxins. The dead cultures administered by the mouth produce no effect unless the intestinal epithelium is injured, in which case poisoning may result. Pfeiffer found that the toxic bodies were to a great extent destroyed at 60° C., but even after heating at 100° C. a small proportion of toxin remained, which had the same action. Later, A. Macfadyen found that the product obtained by grinding up the organisms frozen by means of liquid air, had a very high degree of toxicity when injected intravenously. Like Pfeiffer, he found that the " endotoxin " was in great part destroyed at 60° C.

On the other hand, certain observers (Petri, Ransom, Klein, and others) have obtained toxic bodies from *filtered cultures*. Metchnikoff, E. Roux, and Taurelli-Salimbeni have demonstrated the formation of such diffusible toxic bodies in fluid media. By means of cultures placed in collodion sacs in the peritoneum of animals, they found that the living organisms produce bodies which diffuse through the wall of the sac and cause toxic symptoms. By greatly increasing the virulence of the organism, then growing it in bouillon and filtering the cultures on the third and fourth day, they obtained a fluid which was highly toxic to guinea-pigs (the fatal dose usually being 0·2 c.c. per 100 grams weight). The symptoms closely resemble those obtained by Pfeiffer. They found that the toxicity of the filtrate was not altered by boiling—apparently this toxic substance is different from Pfeiffer's endotoxin. Huntemüller obtained from various strains an acutely acting extracellular toxin which was very labile and which he believed to be identical with the hæmolysin. He obtained an antitoxin to this toxin. The diversity in the results obtained by various workers seems only explicable on the assumption that different strains vary greatly as regards production of extra-cellular toxin.

Immunity.—As this subject is discussed in Chapter VI., only a few facts will be here stated, chiefly for the purpose of making clear what follows with regard to the means of distinguishing the cholera vibrio from other organisms. The guinea-pig or any other animal may be easily immunised against the cholera organism by repeated injections (conveniently made into the peritoneum) of non-fatal doses of dead vibrios; later, the living organisms may be used. In this way a high degree of immunity against the organism is developed; and further, the blood serum of an animal thus immunised (anti-cholera serum) has markedly protective power when injected, *e.g.* intraperitoneally,

even in a small quantity, into a guinea-pig along with five or ten times the fatal dose of the living organism. Under these circumstances the vibrios undergo a granular transformation and, ultimately, solution ; this phenomenon is generally known as Pfeiffer's reaction, and was applied by him to distinguish the cholera vibrio from organisms resembling it. The following are the details :

Pfeiffer's Reaction —A loopful (2 mgrms) of a recent agar culture of the organism to be tested is added to 1 c.c. of ordinary bouillon containing 0·001 c c. of anti-cholera serum. The mixture is then injected into the peritoneal cavity of a young guinea-pig (about 200 grams in weight), and the peritoneal fluid of this animal (conveniently obtained by means of a 1 c.c. syringe) is examined microscopically after a few minutes. If the organisms injected are cholera vibrios, it will be found that they become motionless, swell up into globules, and ultimately break down and disappear—*positive result.* If they are found active and motile, then the possibility of their being true cholera organisms may be excluded—*negative result.* In the former case (positive result) there is, however, still the possibility that the organism has been destroyed by the normal peritoneal fluid A control experiment should be made with 0 001 c.c of normal serum in place of the anti-cholera serum. If no alteration of the organism occurs with its use, then the conclusion is that a true reaction has been given Corresponding bacteriolytic effects may be obtained by *in vitro* methods, introduced since Pfeiffer's original method.

The serum of an animal immunised by the above method has also marked agglutinating and other antibacterial properties (p. 195) against the cholera vibrio, and these properties closely correspond with Pfeiffer's reaction as regards specificity. For the serological identification of V. choleræ the agglutination reaction is now used as in the case of B. typhosus. Such a serum has, however, little protective effect against the toxic action of the dead vibrios, and Pfeiffer maintained that little or no antitoxin to the endotoxin can be produced. On the other hand, Macfadyen, by injecting the endotoxin derived from the vibrios by grinding, obtained a serum which had antitoxic as well as agglutinative and bacteriolytic properties. Metchnikoff and others also obtained antitoxic sera which acted on the extracellular toxins.

The *serum of cholera convalescents* has been found to possess protective and increased bactericidal action. These properties of the serum may be present eight or ten days after the attack of the disease, but are most marked four weeks after ; they then gradually diminish. Specific agglutinins appear in the serum of cholera patients, as in other diseases. They are most

marked in convalescence, reaching the maximum in from two to three weeks from the onset of the disease, the serum then agglutinating in a dilution of 1 : 400 or even 1 : 1000 (Greig). Agglutinins are also often present in the blood of carriers. It should, however, be noted that normal serum may sometimes have an agglutinating effect on the cholera organism in dilutions up to 1 : 20.

For therapeutic purposes several so-called antisera which are supposed to be antitoxic as well as antibacterial have been used. Reports regarding the effects of these sera are of conflicting character, but it cannot be said that they have a markedly beneficial action. The possession of marked antitoxic properties by these sera has not been established experimentally.

Allied Organisms.—*El Tor Vibrio.*—Up till recent times there had been cultivated, from sources other than cholera cases, no organism which gave all the cultural and serum tests (agglutination and Pfeiffer's reaction) of the cholera vibrio. In 1905, however, Gotschlich obtained six different strains of a vibrio which conformed in all these respects. The organisms were obtained at El Tor from the intestines of pilgrims who had died with dysenteric symptoms, and there were no cases of cholera in the vicinity. The organisms in question, however, differed from the classical cholera organism in having marked hæmolytic action, and also in producing a rapidly acting extracellular toxin. There has been some difference of opinion as to whether these organisms are to be regarded as a distinct species or as true cholera vibrios. In view, however, of what we know of variations in the type of the cholera organism, the latter possibility is probably the case.

Paracholera Vibrios.—More recent observations have shown that there occur groups of cases with choleraic symptoms or merely diarrhœa, in which the vibrios present differ in certain respects from the cholera organism. Such cases have been studied by various workers (Castellani, Chalmers and Waterfield, Mackie and Storer), and the term paracholera has been applied. To speak generally, the symptoms are milder than those of true cholera, fatal results being comparatively rare, and the infection does not tend to spread as an epidemic. In addition to those suffering from the disease, similar organisms have been obtained from the stools of contacts—that is, carriers occur. In those affected, the vibrios are often present in the stools in large numbers, and on isolation are found to have the morphological and cultural characters of the cholera organism ; they are also virulent to the guinea-pig on intraperitoneal in-

jection, and to the rabbit on intravenous injection, as in the case of the classical V. choleræ. They are, however, markedly hæmolytic, when tested both on blood agar plates and with suspensions of red corpuscles. Further, they differ serologically from the cholera organism—they are not agglutinated by an anti-cholera serum and they react negatively in Pfeiffer's reaction. They also differ serologically amongst themselves, and several varieties may be distinguished in this way (Mackie). In this group of organisms, producing relatively a mild form of disease, we have manifestly a close analogy to the case of the paratyphoid bacilli.

Anti-Cholera Inoculation.—Preventive inoculation against cholera has attracted attention largely as the result of Haffkine's work in India. On the analogy of Pasteur's anti-anthrax inoculation, injections of attenuated organisms were administered first of all, and were followed by the injection of cultures of organisms of exalted virulence which had been passed repeatedly through the peritoneal cavity of guinea-pigs. Killed suspensions of the organism prepared from cultures on agar have more recently been extensively used as vaccines by Kolle and others. They are prepared according to the general technique described on p. 139. Importance attaches to employing strains of V. choleræ derived from cases in the region in which those who are vaccinated will be stationed. Adequate dosage of the vaccine is also essential. Preventive inoculation has been carried out in India and in Russia, and the results have established its efficiency, both the case incidence and the mortality being reduced. The effect of the preventive inoculation of troops exposed to cholera infection in time of war, both in reducing very greatly the incidence and also in lessening the mortality of the disease, has been strikingly illustrated (Savas, Cantacuzène).

Methods of Diagnosis.—In the first place, the stools ought to be examined microscopically. Dried film preparations should be made and stained by any ordinary stain, though carbol-fuchsin diluted four times with water is specially to be recommended. Hanging-drop preparations should also be made, by which method the motility of the organism can be readily seen. By microscopic examination the presence of vibrios will be ascertained, and an idea as to their number obtained. In some cases the cholera vibrios are so numerous in the stools that a picture is presented which is obtained in no other condition, and a microscopic examination may be sufficient for practical purposes. According to Koch, a diagnosis was made in 50 per cent of the cases during the Hamburg epidemic by microscopic examination alone. In the case of the first appearance of a cholera-like disease, however, all the other tests should be applied before a definite diagnosis of cholera is made.

If the organisms are very numerous, plates of Dieudonné's medium (p. 56) may be inoculated at once and a pure culture obtained from one af the colonies.

If the vibrios occur in comparatively small numbers, the best method is to inoculate peptone solution (1 per cent.) standardised neutral to phenolphthalein, *i.e.* alkaline to litmus, and incubate for from six to eight hours. At the end of that time the vibrios will be found on microscopic examination in large numbers in the surface pellicle, and thereafter plate cultures can readily be made on Dieudonné's medium. If the vibrios are very few in number, the peptone solution which has been inoculated should be examined at intervals till vibrios are found microscopically. A second tube of peptone solution should then be inoculated, and possibly again a third from the second, and then subcultures may be made on Dieudonné's plates. Though Dieudonné's medium in virtue of its alkalinity restrains the growth of most other intestinal bacteria and thus yields, as a rule, a practically pure growth of V. choleræ, in all cases the purity of the strain must be ensured by isolating from single colonies.

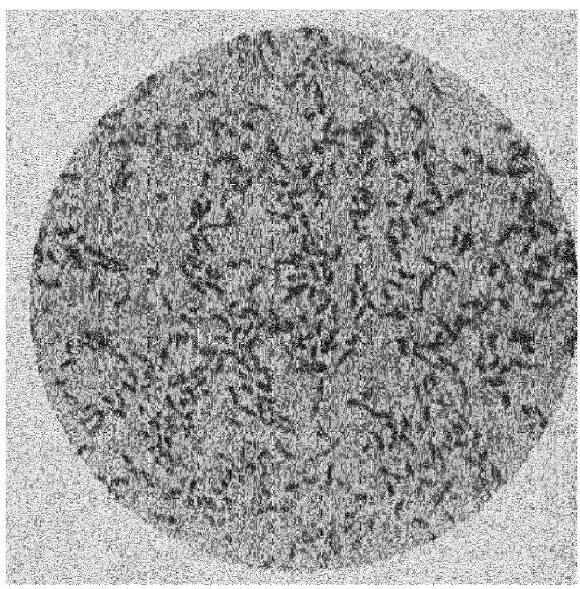


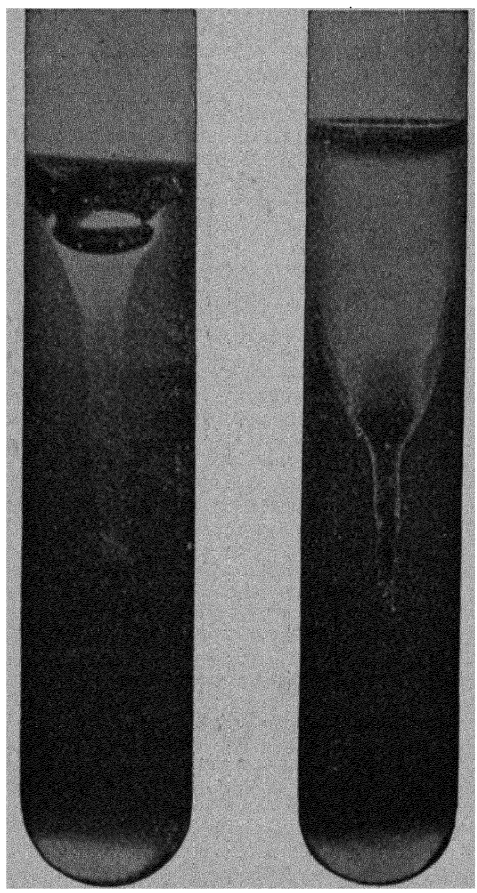
FIG. 116. — Vibrio metchnikovi, both in curved and straight forms; from an agar culture of twenty-four hours' growth. Stained with weak carbol-fuchsin. ×1000.

When a vibrio has been obtained in pure condition by these methods it should be tested, as regards agglutination, with a high titre anti-cholera serum. If it reacts positively it may be accepted for practical purposes as the cholera organism. At the same time the cultural characters and the hæmolytic and pathogenic properties may be tested. If it reacts negatively with anti-cholera serum it may be one of the paracholera group, and similar tests should be made.

A number of other vibrios have been cultivated, which are of interest on account of their points of resemblance to the cholera organism, though probably they produce no pathological conditions in the human subject.

Organisms resembling V. Choleræ—The cholera vibrio belongs to a group of organisms which resemble it closely in microscopic and cultural characters. Some of these have been found to produce disease in animals, *e.g. V. metchnikovi* isolated from an epidemic disease of fowls; some again have been recovered from water or materials suspected of harbouring the cholera organism, *e.g.* Finkler and Prior's vibrio obtained from decomposing fæces from a case of

cholera nostras; while others have been derived from various sources, *e.g.* Deneke's vibrio recovered from cheese. Before the importance of serological tests for the identification of V. choleræ was established, various cultural and biological characters were relied on for distinguishing them. Thus while Metchnikoff's vibrio (Figs. 116, 117, A) closely resembles the cholera organism both in cultural appearances and in giving the cholera-red reaction, it can be readily distinguished from the latter by the effects of inoculation in animals, especially



A B

FIG. 117.—Puncture cultures in peptone-gelatin.

A. Metchnikoff's vibrio. Five days' growth.
B. Finkler and Prior's vibrio. Four days' growth.
Natural size.

in pigeons and guinea-pigs. Subcutaneous inoculation of small quantities of pure culture in pigeons is followed by septicæmia, which produces a fatal result usually within twenty-four hours. Inoculation with the same quantity of the cholera vibrio produces practically no result; even with large quantities death is rarely produced. Metchnikoff's vibrio produces somewhat similar effects in the guinea-pig to those in the pigeon, subcutaneous inoculation being followed by extensive hæmorrhagic œdema and a rapidly fatal septicæmia. Young fowls can be infected by feeding with virulent

cultures. An organism which is apparently the same as the Vibrio metchnikovi was cultivated by Pfuhl from water, and named V. nordhafen. Finkler and Prior's (Figs. 117 B, 118) and Deneke's

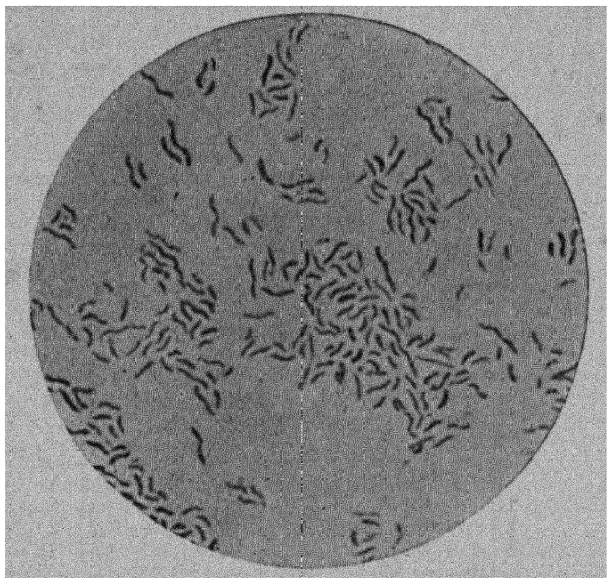


FIG. 118.—Finkler and Prior's vibrio; from an agar culture of twenty-four hours' growth. Stained with carbol-fuchsin. ×1000.

organisms liquefy gelatin rapidly and do not give the cholera-red reaction. Certain other vibrios again are phosphorescent in culture growing at 22° C. None of these organisms are agglutinated by an anti-cholera serum nor do they give Pfeiffer's reaction, and there is no evidence that they have any causal relationship to cholera.

CHAPTER XVII

DIPHTHERIA

THERE is no better example of the valuable contributions of bacteriology to scientific medicine than that afforded in the case of diphtheria. Not only has research supplied a means of distinguishing true diphtheria from conditions which resemble it, but the study of the toxins of the bacillus has explained the manner by which the pathological changes and characteristic symptoms of the disease are brought about, and has led to the discovery of the most efficient means of treatment, namely, anti-diphtheritic serum.

Historical.—Bretonneau, from clinical and pathological observations, distinguished diphtheria as a specific disease; but the proof of this was effected by the discovery of the specific causal organism. The first account of the bacillus now known to be the cause of diphtheria was given by Klebs in 1883, who described its characters in the false membrane, but made no cultivations. It was first cultivated by Löffler from a number of cases of diphtheria, his observations being published in 1884, and to him we owe the first account of its characters in cultures and some of its pathogenic effects on animals. The organism is for these reasons known as the Klebs-Löffler bacillus, or simply as Löffler's bacillus. By experimental inoculation with the cultures obtained, Löffler was able to produce false membrane on damaged mucous surfaces, but he hesitated to conclude definitely that this organism was the cause of the disease, for he did not find it in all the cases of diphtheria examined, he was not able to produce paralytic phenomena in animals by its injection, and, further, he obtained the same organism from the throat of a healthy child. The organism became the subject of much inquiry, but its relationship to the disease may be said to have been definitely established by the brilliant researches of Roux and Yersin, which showed that the most important features of the disease could be produced by means of the separated toxins of the organism. Their experiments were published in 1888–90.

General Facts.—Without giving a description of the pathological changes in diphtheria, it will be well to mention the outstanding features which ought to be considered in connection with its bacteriology. In addition to the formation of false

membrane, which may prove fatal by mechanical effects, the chief clinical phenomena are the symptoms of general poisoning, great muscular weakness, tendency to syncope, and albuminuria; also the striking paralyses which occur later in the disease, and which may affect the muscles of the pharynx, larynx, and eye, or less frequently the lower limbs (being sometimes of paraplegic type), all these being grouped together under the term "post-diphtheritic paralysis." It may be stated here that all these conditions have been experimentally reproduced by the action of the bacillus of diphtheria, or by its toxins. Other bacteria are, however, concerned in producing various secondary inflammatory complications in the region of the throat, such as ulceration, gangrenous change, and suppuration, which may be accompanied by symptoms of general septic poisoning. The detection of the bacillus of Löffler in the false membrane or secretions of the mouth is to be regarded as supplying the only certain means of diagnosis of diphtheria.

Bacillus Diphtheriæ (*Corynebacterium diphtheriæ (Klebs-Löffler)*).—*Microscopical Characters.*—As seen in young cultures the B. diphtheriæ occurs in the form of straight or, more frequently, slightly curved rods, which measure usually about 3–4 μ in length and about 0·5 μ in thickness; both shorter and longer forms, however, are met with. The bacilli vary in configuration, being rounded or tapered at their ends, and they stain unequally, the staining occasionally giving a sort of barred marking (Figs. 119 and 120). They contain granules which produce a beaded appearance and which with certain dyes give a metachromatic reaction, for example, staining a purplish tint with polychrome methylene-blue. They are stained a deep, almost black, colour with Neisser's and other similar stains. These granules are usually situated at the poles of the bacilli—"polar granules"—but occur

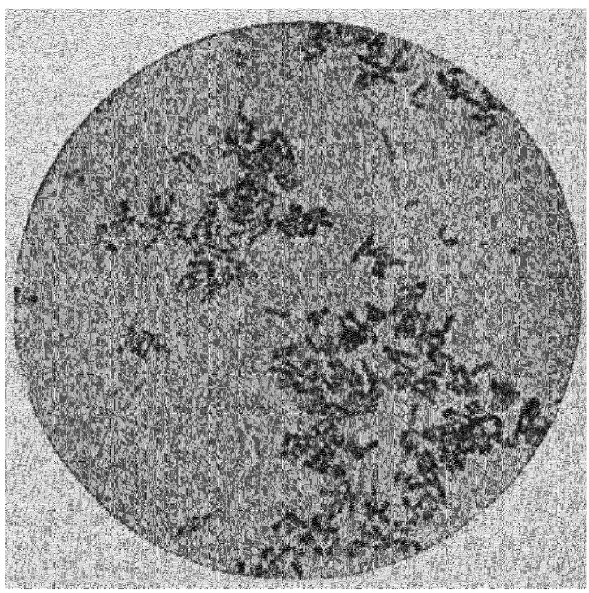


FIG. 119.—Diphther[illegible] a twenty four hours' culture on agar. Stained with methylene-blue. ×1000.

in other parts of the substance, and the longer bacilli may contain several. The ends of the bacilli are often swollen, especially in the longer forms; later these may form club-like structures which stain deeply, while the protoplasm becomes broken up into globules. Other bacilli may become thicker and segmented and various stages of disintegration are seen (Fig. 121). These aberrant changes are usually regarded as the result of involution. Occasionally branched forms are met with. A characteristic feature in a film is the arrangement of the bacilli. They lie at various angles to one another, giving an appearance which has been compared to Chinese letters or cuneiform characters. This is apparently due to the snapping mode of division (p. 5); when a bacillus undergoes division the process at first occurs at one side and the two new individuals remain attached and set at an angle. It should be recognised that the size and general appearance vary with different strains of organisms and with different media, as well as with the duration of the growth. Sometimes quite short types are met with.

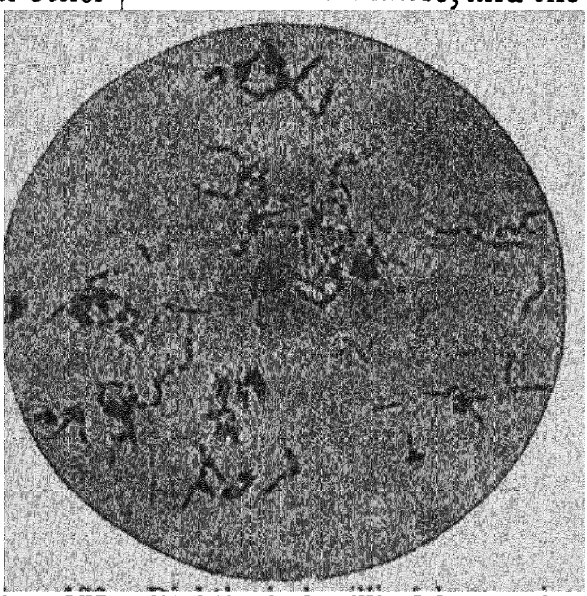


FIG. 120.—Diphtheria bacilli of larger size than in previous figure, showing also irregular staining of protoplasm. From a three days' agar culture. Stained with weak carbol-fuchsin. ×1000.

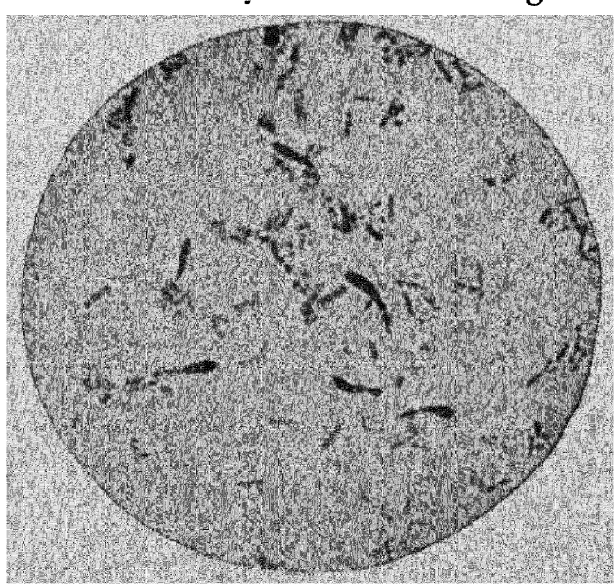


FIG. 121.—Involution forms of the diphtheria bacillus; from an agar culture of seven days' growth. See also Plate III., Fig. 13. Stained with carbol-thionin-blue. ×1000.

In films from the pharynx or from the membrane the bacilli have the same general characters, but they tend on the whole to stain more evenly and granules are not so pronounced (Fig. 124).

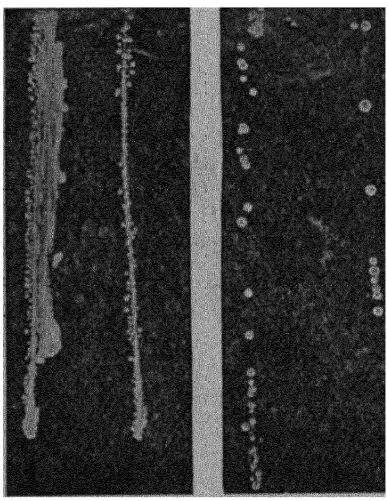
Staining.—They take up the basic aniline dyes, *e.g.* methylene-blue in watery solution, with great readiness, and stain deeply, the granules often giving the metachromatic reaction as described. They are Gram-positive, though they are rather more easily decolorised than the pyogenic cocci. By Neisser's stain (p. 110) the bacilli are seen to contain granules stained almost black, the rest of the bacillary substance being yellowish-brown, or, by the erythrosin method, pink (Plate III., Fig. 12). In applying the stain a serum culture of eighteen to twenty-four hours' growth ought to be used. The granules brought out by Neisser's method are often not visible in a methylene-blue preparation.

Neisser's stain is undoubtedly an important auxiliary in the recognition of the diphtheria bacillus, but the results of its use are to be interpreted with caution. Granules staining black are not peculiar to the diphtheria bacillus. Some cocci, often giving a metachromatic reaction with methylene-blue, may be stained black; and other bacilli may contain such granules. A not uncommon organism with such a character occurring in the throat is a streptobacillus with square ends; it has no resemblance to the diphtheria bacillus in a methylene-blue preparation, but when stained by the Neisser method may give an appearance very like that organism. On the other hand, a culture of Hofmann's pseudo-diphtheria bacillus (p. 482) reacts negatively with Neisser's stain: at most a few scattered granules may occur in the preparation, but the bacilli have not the beaded appearance. It will be found a good working plan to use the Neisser stain only after finding bacilli in a film from a serum culture stained by methylene-blue, which present the features of the diphtheria bacillus Such bacilli should react positively to the Neisser stain before being accepted as such. The stain is of special service in the case of the smaller forms of the diphtheria bacillus, the details of whose structure are imperfectly differentiated by methylene-blue. And again when the diphtheria bacilli are scanty they may be overlooked in a methylene-blue preparation, whereas they are more readily detected in a Neisser preparation.

All true diphtheria bacilli give the characteristic appearance with the Neisser stain, but it is of importance to observe that some hard waters interfere with the reaction. In such circumstances distilled water ought always to be used for washing the preparations.

Cultivation.—The diphtheria bacillus grows best in cultures at the temperature of the body; growth still takes place at 22° C., but practically ceases about 20° C. The best media are those

which contain serum, *e.g.* Löffler's original medium (p. 53), but growth occurs also on the ordinary agar media. If inoculations be made on the surface of solidified serum with a piece of diphtheria membrane, colonies of the bacillus may appear in twelve hours, and are well formed within twenty-four hours, often before any other growths are visible. The colonies are small circular discs of opaque whitish colour, their centre being thicker and of darker greyish appearance, when viewed by transmitted light, than the periphery. Their margins are at first regular, but later they become wavy or even crenated. On the second or third day they may reach 3 mm. in size, but when numerous they remain smaller. Their surface becomes distinctly granular, and when there is a mass of growth it comes to have a yellowish tint. On the agar media the colonies have much the same appearance (Fig. 122) but grow less quickly, and sometimes they may be comparatively minute, so as rather to resemble those of the streptococcus pyogenes. In stroke cultures the growth forms a continuous layer of the same dull whitish colour, the margins of which often show single colonies partly or completely separated. On *gelatin* at 22° C. a puncture culture shows a line of dots along the needle track, whilst the growth at the surface is only slight. In none of the media does any liquefaction occur. Growth occurs under anaerobic as well as aerobic conditions. In *bouillon* the organism produces a turbidity which soon settles to the bottom and forms a powdery layer on the wall of the vessel. If the growth is started on the surface and the flask is kept at rest, a distinct scum forms, and this is especially suitable for the development of toxin.



a *b*

FIG. 122.—Cultures of the diphtheria bacillus on an agar plate; twenty-six hours' growth. (Natural size.)

(*a*) Two successive strokes; (*b*) isolated colonies from the same plate.

Ordinary bouillon becomes acid during the first two or three days, and several days later again acquires an alkaline reaction which may then rise above the original. Changes in reaction are most pronounced when the medium contains sugars fermentable by the organism, but even in sugar-free media reversal of the reaction occurs to come extent. This is due to

formation of carbonates. Peptone has a buffering action and diminishes the change to the acid side.

Fermentative Properties.—The action of the B. diphtheriæ in fermentation has been extensively studied and is of considerable importance. It may be said that the organism produces acid *without gas formation* from glucose, maltose, and galactose, and no acid from saccharose and mannite; the action on lactose is somewhat indefinite. Glucose and saccharose are the sugars employed for practical purposes. In carrying out the tests it is important to ensure that there is abundant growth in the medium, and for this reason a serum medium such as Hiss's (p. 64) is to be recommended, litmus or some other indicator being added. In Hiss's medium the development of acid is attended by the formation of clot. Reference to the fermentative properties of allied organisms is made below (p. 481).

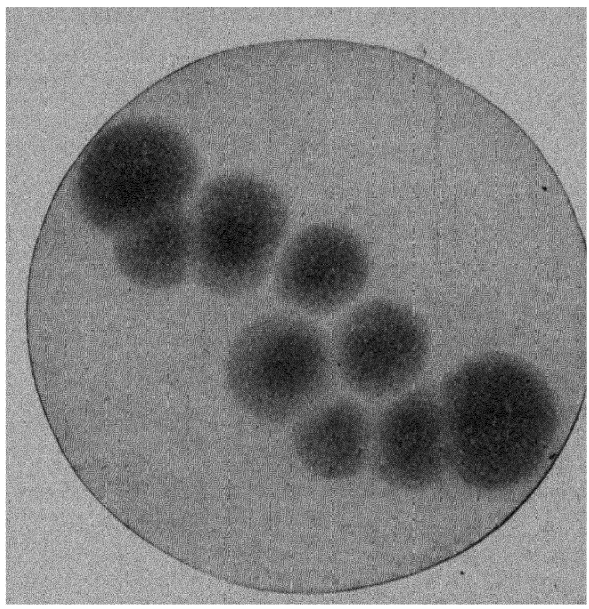


FIG. 123.—Diphtheria colonies, two days old, on agar. ×8.

Powers of Resistance, etc.—In cultures the bacilli possess long duration of life; at room temperature they may survive for two months or longer. In the moist condition, whether in cultures or in membranes, they have a low power of resistance, being killed at 60° C. in a few minutes. On the other hand, in the dry condition they have great resisting powers. In membrane which is perfectly dry, for example, they can resist a temperature of 98° C. for an hour. Dried diphtheria membrane, kept in the absence of light and at the room temperature, has been proved to contain diphtheria bacilli still living and virulent at the end of several months. The presence of light, moisture, or a higher temperature causes them to die out more rapidly. Corresponding results have been obtained with bacilli taken from cultures and kept on dried threads. These facts, especially with regard to drying, are of importance, as they show that the contagium of diphtheria may be preserved for a long time in the dried condition.

Distribution of the Bacillus.—The B. diphtheriæ may be found in the membrane wherever it is formed, and also occurs in the secretions of the pharynx and larynx in the disease. It may be mentioned that distinctions formerly drawn between true diphtheria and non-diphtheritic conditions from the appearance and site of the membrane have no scientific value, the only true criterion being the presence of the diphtheria bacillus. The

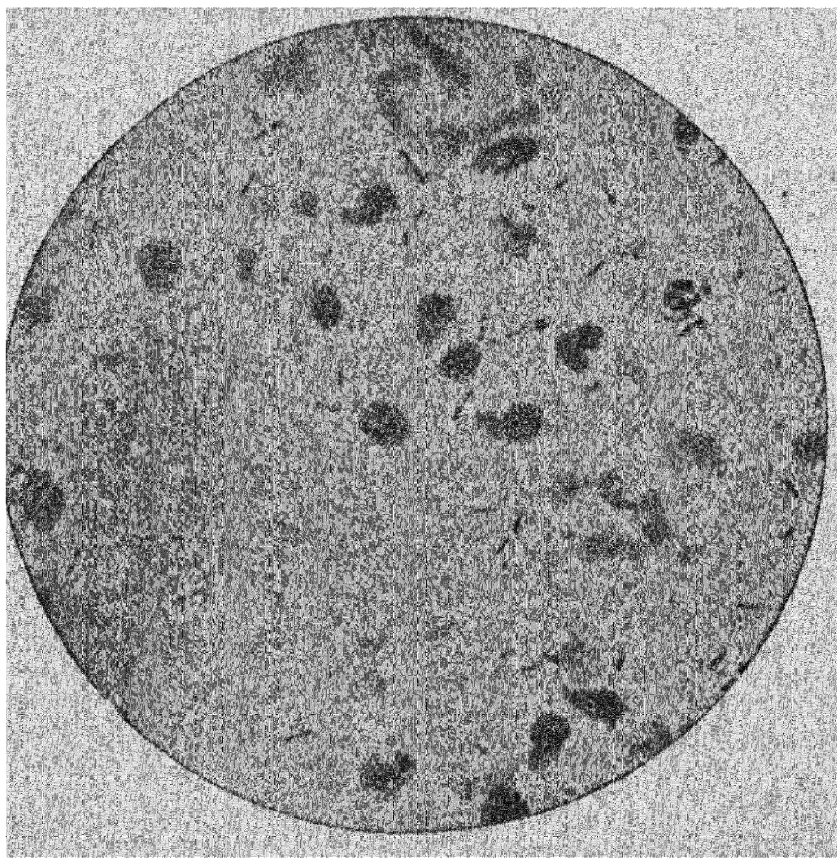


FIG. 121.—Film preparation from diphtheria membrane, showing numerous diphtheria bacilli. One or two degenerated forms are seen near the centre of the field. (Cultures made from the same piece of membrane showed the organism to be present in practically pure condition.)

Stained with methylene-blue. ×1000.

occurrence of a membranous formation produced by streptococci has already been mentioned (p. 250). Virulent diphtheria bacilli have been found in a considerable proportion of cases of fibrinous rhinitis. In the case of any nasal lesion, however, the test for virulence should always be made, as diphtheria-like bacilli without virulence are of comparatively common occurrence.

In diphtheria the membrane has a somewhat different structure, according as it is formed on a surface covered with stratified

squamous epithelium, as in the pharynx, or on a surface covered by ciliated epithelium, as in the trachea. In the former situation necrosis of the epithelium occurs either uniformly or in patches, and along with this there is marked inflammatory reaction in the connective tissue beneath, attended by abundant fibrinous exudation. The necrosed epithelium becomes raised up by the fibrin, and its interstices are also filled by it; fibrinous exudation also occurs around the vessels in the tissue beneath. The membrane is thus firmly adherent, and when artificially detached it leaves a

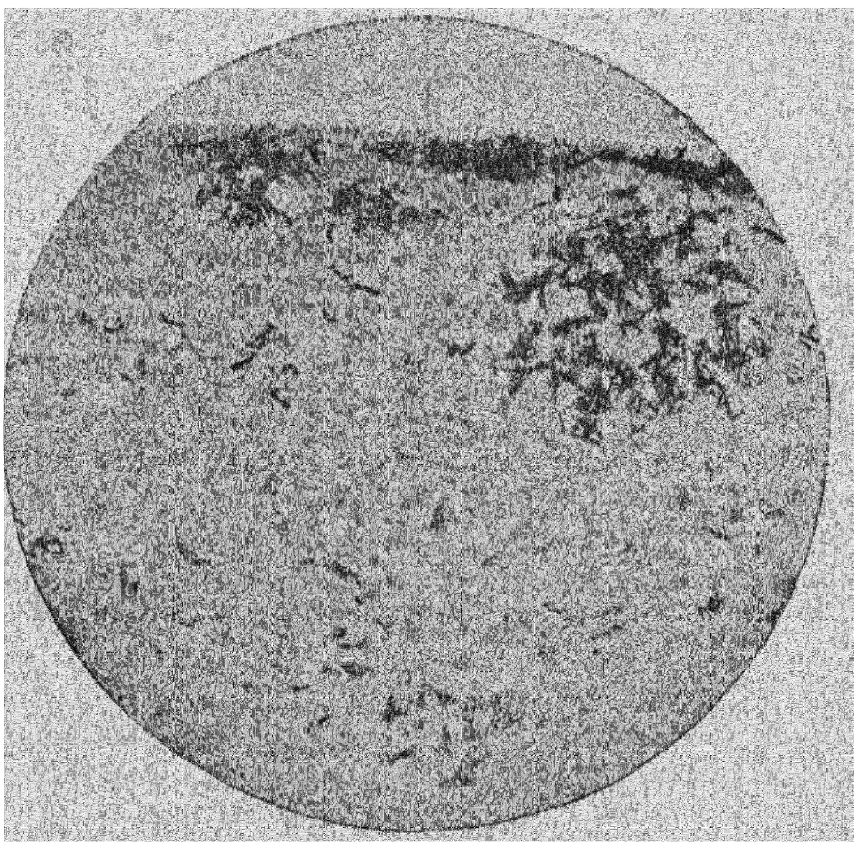


FIG. 125.—Section through a diphtheritic membrane in trachea, showing diphtheria bacilli (stained darkly) in clumps, and also scattered amongst the fibrin. Some streptococci are also shown towards the surface on the left side.
Stained by Gram's method and Bismarck-brown. ×1000.

bleeding surface. In the trachea, on the other hand, the epithelial cells rapidly become shed, and the membrane is found to consist almost exclusively of fibrin with leucocytes, the former arranged in a reticulated or somewhat laminated manner, and varying in density in different parts. The membrane lies upon the basement membrane, and is comparatively loosely attached.

The position of the diphtheria bacilli varies somewhat in different cases, but they are most frequently found lying in oval or irregular clumps in the spaces between the fibrin, towards the

superficial, that is, usually, the oldest part of the false membrane (Fig. 125). There they may be in a practically pure condition, though streptococci and occasionally some other organisms may be present along with them. They may occur also in deeper parts, but are rarely found in the fibrin around the blood vessels. On the surface of the membrane they may be also seen lying in large numbers, but are there accompanied by numerous other organisms. Occasionally a few bacilli have been detected in the lymphatic glands. As Löffler first described, they may be found after death in pneumonic patches in the lung, these being due to a secondary extension by the air passages. They have also been occasionally found in the spleen, liver, and other organs after death ; in a few instances they have been cultivated from the blood during life. Such occurrences are probably to be explained by an entrance into the blood stream shortly before death. It may be accepted, however, that the growth of the organism is essentially local, that it does not invade the tissues to any extent, and that its important effects are produced by toxins absorbed from the site of growth. The B. diphtheriæ may also infect other mucous membranes. It is found in true diphtheria of the conjunctiva, and may also occur in similar affections of the vulva and vagina. In most of the recorded cases of such lesions, however, the identity of the bacilli has not been established ; some of them are described as responding to treatment with antitoxin. During the war virulent diphtheria bacilli were cultivated from wounds in a small proportion of cases ; their presence was not attended by any special effects.

Association with other Organisms.—In diphtheria the pyogenic organisms—streptococci, staphylococci, pneumococci, etc.—are practically always present in the pharynx, occurring there in varying numbers and combinations. Hæmolytic streptococci are, however, relatively abundant in certain cases and can hardly be without effect in aggravating the condition. They are often found lying side by side with the diphtheria bacilli in the membrane, and also penetrating more deeply into the tissues. In some cases of tracheal diphtheria we have found streptococci alone at a lower level in the trachea than the diphtheria bacilli, where the membrane was thinner and softer, the appearance in these cases being as if the streptococci acted as exciters of inflammation and prepared the way for the bacilli. Further, some of the complications of diphtheria are due to the action of pyogenic organisms. The extensive swelling of the tissues of the neck, sometimes attended by suppuration in the glands, and also various hæmorrhagic conditions, have been

found to be associated with their presence; the diphtheritic lesion enables them to get a foothold in the tissues, where they exert their usual action and may lead to extensive suppurative change, to septic poisoning, or even to septicæmia. In cases where a gangrenous process is superadded, a great variety of organisms may be present, some of them being anaerobic. Against such complications produced by other organisms anti-diphtheria serum produces no effect.

Effects of Inoculation.—In considering the effects produced in animals by experimental inoculations of pure cultures, we have to keep in view the local changes which occur in diphtheria, and also the symptoms of general poisoning.

As Löffler stated in his original paper, inoculation of the healthy mucous membranes of various animals with pure cultures causes no lesion, but the formation of false membrane may result when the surface is injured by scarification or otherwise. A similar result may be obtained when the trachea is inoculated after tracheotomy has been performed. The membrane produced by such experiments is usually less firm than in human diphtheria, and the bacilli in the membrane are less numerous. Rabbits inoculated after tracheotomy often die, and Roux and Yersin were the first to observe that in some cases paralysis might appear before death.

Subcutaneous injection in guinea-pigs of diphtheria bacilli in a suitable dose produces death within thirty-six hours. At the site of inoculation there is usually a small patch of greyish membrane with some necrosis, whilst in the tissues around there is extensive inflammatory œdema, often associated with hæmorrhages, and there is also swelling of the corresponding lymphatic glands. The internal organs show general congestion, the suprarenal capsules being especially reddened and often hæmorrhagic. The renal epithelium may show cloudy swelling, and there is often effusion into the pleural cavities. After injection the bacilli increase in number for a few hours, but multiplication soon ceases, and at the time of death they may even be less numerous than when injected. The bacilli remain practically local, cultures made from the blood and internal organs usually giving negative results, though sometimes a few colonies may be obtained. If a non-fatal dose of a culture be injected, a local necrosis of the skin and subcutaneous tissue may follow at the site of inoculation.

In rabbits, after subcutaneous inoculation, results of the same nature follow, but these animals are less susceptible than guinea-pigs, and the dose requires to be proportionately larger. Roux

and Yersin found that after intravenous injection the bacilli rapidly disappeared from the blood, and when 1 c.c. of a broth culture had been injected no trace of the organisms could be detected by culture after twenty-four hours; nevertheless the animals died with symptoms of general toxæmia, nephritis also being often present. The dog and sheep are also susceptible to inoculation with virulent bacilli, but the mouse and rat enjoy a high degree of immunity.

An *intracutaneous method* of injection has been found to be of service in testing the virulence, and thus in the identification, of the diphtheria bacillus, especially when used in conjunction with the injection of antitoxin. Injection with a fine syringe of a small amount of diphtheria bacilli into the superficial part of the skin in a guinea-pig produces a circumscribed swelling which is followed by superficial necrosis in from one to two days; whereas, if the animal has received previously an injection of, say, 250 units of antitoxin, the result is negative. The result is also negative (without antitoxin) in the case of an avirulent diphtheroid.

The following is the method as modified by Zingher and Soletsky. A twenty-four hours' growth on a tube of Loffler's serum is emulsified in 20 c.c. of normal saline, and of this 0·15 c.c. is injected intracutaneously into the abdominal wall of a guinea-pig. Four or even six injections of different strains can be carried out at the same time in the same animal. Similar injections are made in an animal previously treated with about 250 units of antitoxin, the antitoxin being introduced intracardially immediately before, or intraperitoneally twenty-four hours before. In the case of the B. diphtheriæ any positive reaction in the first animal should be accompanied by a negative result in the control as a result of the antitoxin. It is advisable to control the actual number of bacilli, *e.g.* by an opacity test as recommended by Eagleton and Baxter, ten million bacilli being used for each injection.

The Toxins of the B. Diphtheriæ.—As in the above experiments the symptoms of poisoning, and ultimately a fatal result, occur when the bacilli are not increasing in number, or even after they have practically disappeared, Roux and Yersin inferred that the chief effects were produced by toxins, and this supposition they proved to be correct. They showed that broth cultures of three or four weeks' growth freed from bacilli by filtration were highly toxic. The filtrate when injected into guinea-pigs and other animals produces practically the same effects as the living bacilli; locally there is little fibrinous exudation but a considerable amount of inflammatory œdema, and, if the animal survive long enough, necrosis of the super-

ficial tissues in varying degree may follow. The toxicity may be so great that 0·002 c.c. or even less may be fatal to a guinea-pig in five days.

After injection either of the toxin or of the living bacilli, when the animals survive long enough, paralytic phenomena occasionally occur. The hind-limbs are usually affected first, the paralysis afterwards extending to other parts, though sometimes the fore-limbs and neck first show the condition. Sometimes symptoms of paralysis do not appear till two or three weeks after inoculation. After paralysis has appeared, a fatal result usually follows in the smaller animals, but in dogs recovery may take place. There is evidence that these paralytic phenomena are produced by *toxone*, as they specially occur when there is injected along with the toxin sufficient antitoxin to neutralise the more rapidly acting toxin proper. This toxone is supposed by Ehrlich to have a different toxic action, *i.e.* a different toxophore group (p. 173), from that of the ordinary toxin; it produces the late nervous phenomena, while its local action on the tissues is very slight. It also has a weaker affinity for antitoxin, and thus much of it may be left unneutralised. It is to be noted in this connection that paralytic symptoms are of not uncommon occurrence in the human subject after treatment with antitoxin, the explanation of which occurrence is probably the same as that just given. One point of much interest is the high degree of resistance to the toxin possessed by mice and rats. Roux and Yersin, for example, found that 2 c.c. of toxin, which was sufficient to kill a rabbit in sixty hours, had no effect on a mouse, whilst of this toxin even $\frac{1}{15}$ c.c. produced extensive necrosis of the skin of the guinea-pig.

Preparation of the Toxin.—The obtaining of a very active toxin in large quantities is an essential in the preparation of anti-diphtheritic serum. Certain conditions favour the development of a high degree of toxicity, namely, *a free supply of oxygen, the presence of a large proportion of peptone or albumin in the medium, the absence of substances which produce an acid reaction, and an initial reaction of* P_H *7·5–8.* In the earlier work a current of sterile air was made to pass over the surface of the medium, as it was found that by this means the period of acid reaction was shortened and the toxin formation favoured. This expedient is now considered unnecessary if an alkaline medium free from glucose is used, and the cultures are made in shallow flasks. The absence of glucose may be attained by keeping the meat for some time at the room temperature before preparing the medium (Spronck). L. Martin introduced a medium composed of equal parts of freshly prepared peptone (by digesting pigs' stomachs with HCl at 35° C.) and glucose-free veal bouillon. By this medium he has obtained a toxin of which 0·002 c.c. is the

fatal dose to a guinea-pig of 500 grams. Another medium which possesses advantages is Hartley's broth (p. 50). Park and Williams and also Dean found that the amount of glucose present in ordinary beef is not sufficient to interfere with toxin formation, provided that a considerable amount of peptone, 2 per cent., be added, and the medium be made sufficiently alkaline; they added to each litre of broth 7 c.c. of normal caustic soda solution. There is in all cases a period at which the toxicity reaches a maximum; Roux and Yersin found this period to be two to three weeks, but by later methods the greatest toxicity is reached about the tenth day or even earlier. An important factor is the relative size of surface of the medium exposed to the air, toxin formation occurring most rapidly in shallow vessels. When maximum toxicity has been reached the clear fluid is decanted from the sediment and covered with a layer of toluol; it is then shaken on several occasions for two or three days. When toxin is to be used for testing purposes it is kept in an ice-chest, for about twelve months, till it has been "ripened" or stabilised; it is then standardised. It may be added that the power of toxin formation varies much in different races of the diphtheria bacillus, and that many may require to be tested ere one suitable is obtained.

Properties and Nature of the Toxin.—The toxic substance in filtered cultures is a relatively unstable body. When kept in sealed tubes in the absence of light, it may preserve its powers little altered for several months, but, on the other hand, it gradually loses them when exposed to the action of light and air. As has been shown, the toxin probably does not become destroyed, but its toxophore group suffers a sort of deterioration, so that a toxoid is formed which has still the power of combining with antitoxin (p. 191). Heating at 58° C. for two hours destroys the toxic properties in great part, but not altogether. When, however, the toxin is evaporated to dryness, it has much greater resistance to heat. One striking fact, discovered by Roux and Yersin, is that after an organic acid, such as tartaric acid, is added to the toxin the toxic property disappears, but it can be in great part restored by again making the fluid alkaline.

Toxic bodies have also been obtained from the tissues of those who have died from diphtheria. Roux and Yersin, by using a filtered watery extract from the spleen in very virulent cases of diphtheria, produced in animals death after wasting and paralysis, and also obtained similar results by employing the urine. The subject of toxic bodies in the tissue was, however, specially worked out by Sidney Martin. He separated from the tissues, and especially from the spleen, of patients who have died from diphtheria, by precipitation with alcohol, chemical substances of two kinds, namely, albumoses (proto- and deutero-, but especially the latter) and an organic acid. The albumoses, when injected into rabbits, especially in repeated doses, produced fever, diarrhœa, paresis, and loss of

weight, with ultimately a fatal result. Martin considered that the toxic effects in the human disease were due to the presence in the membrane of an enzyme which has a proteolytic action within the body, resulting in the formation of poisonous albumoses ; but this has not been established by subsequent work.

Variations in Virulence and in Toxin Production.—The distinction between virulence—the power to invade the tissues and produce disease—and the faculty of toxin production has already been emphasised (p. 472); it is well illustrated in the case of B. diphtheriæ. For comparative tests of virulence known quantities of bacilli from a young culture on solid medium should be used for injecting a guinea-pig, as in fluid cultures the presence of variable amounts of toxin modifies the result. As shown by the amount of culture necessary to produce death of a guinea-pig, it may be said that all degrees of virulence are met with among freshly cultivated strains. The virulence of a given strain, moreover, is usually well maintained in culture. A certain falling off has sometimes been observed after a time. Further, unlike what obtains with other organisms, little or no increase of virulence can be obtained by means of *passage*. The earlier results of Roux and Yersin, according to which after an attack of diphtheria the bacilli in the throat gradually become attentuated and ultimately non-virulent, have not been substantiated ; and conversely, no one has succeeded in transforming a non-virulent diphtheria bacillus into a virulent one. It is now generally accepted that virulence is a relatively stable property. In the ordinary subcutaneous test for virulence (p. 471) a whole growth on a serum slant culture is used, and either death of the animal or no pathogenic effect is the common result. Some strains non-virulent on injection have been found to produce a small amount of toxin in media or to give rise to a small amount of antitoxin on injection. These facts indicate that all transitions are found, and rather suggest that the non-virulent organisms belong to the same species as the virulent.

A certain correspondence, however, exists between virulence and toxin production, inasmuch as every virulent strain produces toxin whilst the non-virulent do not (apart from the exceptions mentioned). As in the case of virulence, all degrees of toxin production are met with, but the two properties do not run in a proportionate manner, as some strains of moderate virulence are very active in toxin production. In the preparation of antitoxins the obtaining of a very powerful toxin is necessary, and for this purpose many strains may have to be

tested before a satisfactory toxin producer is obtained. A strain obtained by Park and Williams many years ago, known as No. 8, has been used very extensively all over the world and is noted for its properties in this respect. This is an illustration of the well-recognised fact that the power of toxin production usually remains unchanged for a long period in conditions of artificial cultivation.

Diphtheria Carriers.—It has been known for some time that the B. diphtheriæ may persist for considerable periods in the throats of those who have suffered from the disease. As a rule, it disappears within a few days after recovery, but in a small proportion of cases it may be found for several weeks or even months afterwards. Such individuals harbouring the bacilli are known as "convalescent carriers." The bacillus may also be found in the throats of individuals who have been in contact with patients or other carriers—"contact carriers," and accordingly these also may spread infection. Some of the carriers suffer from slight indisposition, sore throat, etc., but others have no clinical symptoms at all. It is generally admitted that the persistence of the bacilli is aided by abnormal conditions of the fauces, and the treatment of these is thus of importance. The bacilli have been found, for instance, in the crypts of enlarged tonsils, and the removal of the latter may be attended by favourable results. The carrier condition may be also due to the presence of bacilli in the nasal cavity and here also it may or may not be accompanied by morbid states. In all cases, apart from the actual presence of diphtheria, when an organism possessing the morphological and cultural characters of the B. diphtheriæ has been isolated the test for virulence must be made. It is now generally recognised that the avirulent types play no part in the spread of the disease, and that individuals harbouring them are not of danger to the community. We may add that, as a rule, chronic carriers give a negative Schick reaction, that is, are immune (*vide infra*). In the investigation of healthy contact carriers the Schick test accordingly adds valuable information when a suspicious organism has been cultivated from the fauces. Thus, if the suspected carrier is Schick-positive, *i.e.* non-immune, the organism is likely to be non-virulent.

In one or two exceptional instances, diphtheria bacilli have been obtained from the lower animals, *e.g.* cow and horse, but there is no evidence that animal infection plays any part in the spread of the human disease. It may also be added that the so-called "diphtheritic" diseases of animals are due to quite different organisms.

Immunity.—This is described in the general chapter on Immunity. It is sufficient to state here that a high degree of immunity, against both the bacilli and their toxins, can be produced in various animals by gradually increasing doses either of the bacilli or of their filtered toxins (p. 187). As a result of the immunisation, antitoxins appear in the serum, and these are capable of protecting animals against infection either with diphtheria bacilli or their toxins. They also have curative effects in animals which are already the subjects either of infection or intoxication.

Reference has already been made (p. 188) to the fact that in a certain proportion of horses in the natural state antitoxin is present in the blood, and a similar statement also holds with regard to the human subject. The presence of the antitoxin is shown by mixing a certain amount of the serum with a small quantity of toxin and injecting some of the mixture intracutaneously in the guinea-pig (Römer's test); an injection of the same amount of toxin alone is made at another spot as a control. The application of this test gave important knowledge with regard to the presence of antitoxin at different ages, but for practical purposes it has been superseded by the Schick test which may now be described. It is stated that the presence of $\frac{1}{10}$ unit of antitoxin per cubic centimetre of serum is sufficient to afford the individual protection against infection.

Schick Test.—This test depends upon the fact that when a minute amount of diphtheria toxin is introduced into the skin an inflammatory reaction results, provided that no antitoxin is present in the blood to neutralise the effects. As has just been stated, however, the blood of a certain number of individuals does contain a small amount of antitoxin, and in these the reaction does not occur—the result is negative. The presence of antitoxin in the blood is attended by a certain amount of immunity, and thus the test gives an indication of the presence or absence of immunity, according as the result is negative or positive respectively. The amount of toxin injected is of importance; it should be sufficient to ensure a positive reaction in an individual without antitoxin, and it should not be so great as to be in excess of the amount of antitoxin supposed to be associated with immunity. The dose of toxin used is $\frac{1}{50}$ of a M.L.D. of diphtheria toxin for the guinea-pig. The toxin used is a carbolised filtrate of a bouillon culture of the B. diphtheriæ, and this will keep unchanged for several months. When the test is to be made a small quantity is diluted so that the amount of fluid to be injected, namely, 0·2 c.c., contains the dose of toxin

just mentioned. The diluted toxin ought not to be used after twenty-four hours, as it deteriorates. Occasionally there occur pseudo-reactions of the nature of a supersensitiveness or allergy, which are due to certain substances other than the toxin in the filtrate, this allergy being met with alike in immune and non-immune subjects. Diphtheria toxin is readily destroyed by heating, whereas the substances leading to pseudo-reactions are not. The diluted toxin heated to 75° C. for five minutes can thus be used as a control, and of course any effect produced by it will be of the nature of a pseudo-reaction, that is, will not be due to the true toxin. (Outfits for carrying out this test are now supplied by various firms. These comprise a capillary tube containing 1 M.L.D. of toxin, and a bottle containing 10 c.c. sterile salt solution to make the dilution. The mixture will then contain 50 test doses, each being 0·2 c.c.).

The test is made by intracutaneous injection on the flexor aspect of the forearm, one arm being used for the toxin, the other for the heated toxin as a control. The arm is cleansed with a little spirit and the fluid (0·2 c.c.) is injected from a small syringe with a fine needle, the point of the latter being introduced obliquely through the epidermis. When the injection is satisfactorily made a small localised elevation or wheal results. In the case of a positive reaction (in a non-immune individual without pseudo-reaction) an area of redness and slight swelling appears after twenty-four to forty-eight hours, and reaches its maximum about the fourth day, when it may measure up to 2 cm. or more in diameter. It persists for seven days and then gradually fades, becoming brownish in tint, and there is usually some desquamation of the surface epithelium. The control injection on the other arm is of course attended by no result. If the reaction is negative, no change follows at the site of injection of the diluted toxin, the condition of the two arms being similar.

A pseudo-reaction is evidenced also by an area of redness, but it appears earlier, often after six hours, is less intense and less definite at its margins than the positive reaction, and usually disappears in from one to three days. If it is associated with a negative Schick reaction, the result will be the same on the two arms. If a positive Schick is present in addition, the reaction due to the unheated toxin will be more marked and persist longer than the reaction on the control arm. The difference is, as a rule, specially marked on the fourth to seventh day, when the reading should be taken ; a positive reaction is then still present, while a pseudo-reaction is quite gone. Pseudo-

reactions are most frequently met with in persons over six years of age, and in them a control is absolutely necessary.

Extensive series of observations at different periods of life have been carried out by the Römer and by the Schick method, and the results correspond on the whole pretty closely. It may be said that, at the time of birth, the serum of 80 per cent. of children contains antitoxin, and thus they give a negative Schick reaction, that this number falls for a year or two, at first rapidly, and that then there is a gradual rise until in adult life the original level is reached. The age of maximum susceptibility may be said to be from one to four years. It is generally agreed that the antitoxin of the infant represents a passive immunity transferred from the mother, hence the percentages of mothers and of children whose serum contains antitoxin, correspond. This passive immunity falls off somewhat rapidly, and thus a greater proportion of Schick positive reactions occur. The gradual diminution of the number of the latter which then follows is supposed to be due to a process of active immunisation occurring as the result of the individuals harbouring diphtheria bacilli and to slight infections by the organism.

Toxin-Antitoxin Immunisation.—This is a process of active immunisation by means of a small amount of toxin partly neutralised by antitoxin, the amount of the mixture used for injection (1 c.c.) being only slightly toxic to a guinea-pig (the exact amounts of toxin and antitoxin used still varies in different places). The method has been carried out on a large scale by Park, Zingher, and others, and the results have been distinctly favourable. Three consecutive injections are given with an interval of a week between, and the immunisation is recommended to be carried out in young children from about a year old, so as to give protection during the dangerous period of life. Immunity, as shown by a previously positive Schick reaction becoming negative, develops somewhat slowly, but in 90 per cent. of the cases the reaction becomes negative after three months. Observations were carried out on 200,000 school children in America; half of these were tested for the Schick reaction, and those giving a positive reaction were immunised, the other half were used as controls. The result was that amongst the controls the number of cases of diphtheria which occurred was five times as great as amongst those tested and treated. Further, all the severe cases occurred amongst the controls (Park and Williams).

Therapeutic Effects of Diphtheria Antitoxin.—The use of this antitoxin for the prevention and treatment of diphtheria con-

stituted the first great contribution of bacteriology to practical therapeutics. The practice as to method of administration and dosage still varies, but larger doses are now given than formerly, and it is generally recognised that the best routes of injection are the intramuscular and intravenous. The former is the more convenient, but the latter gives the more rapid distribution of antitoxin throughout the body and is thus more effective. Further, the longer treatment has been delayed or the severer the case, the more is the intravenous method to be preferred and the larger should be the dose. Subcutaneous injection is much less efficient, as the absorption of antitoxin into the circulation takes place relatively slowly. Some authorities give the antitoxin in a single dose, others in two or more doses. Theoretically, there appears to be no advantage in the latter method, as a single dose will keep the antitoxin content of the blood during the disease at a level more than sufficient to neutralise the toxin; nevertheless, some maintain that administration in more than one dose has an advantage. The dose, of course, varies also according to the age of the patient, but for a child is proportionately larger than for an adult. We may say that the average dosage recommended is, for an infant, 2000–10,000 units, varying according to the principles mentioned above, and for an adult, 5000–50,000 units. In some places, however, the practice is to give even larger amounts. It would occupy too much space to state and discuss adequately the results of antitoxin treatment, but it may be said that statistics obtained from all parts of the world afford convincing proof of its advantage. And this is reflected in the practically universal belief of the medical profession in its efficiency.

Other Antibodies.—As in the case of other organisms, the injection into animals of the B. diphtheriæ itself in the living or dead condition, gives rise to anti-bacterial substances—agglutinins, complement-fixing bodies, etc. Some of these may be present in antitoxic sera, due to the presence of disintegration products of the bacilli in the toxin used for preparing the antitoxin; but there is no evidence that such substances play any part in the therapeutic effects. Certain strains of the B. diphtheriæ have been distinguished by various observers according to their agglutinative properties, but no definite recognition of types has resulted, and no facts of practical importance have emerged. It may be added that the strains differing serologically according to agglutination tests all produce a similar toxin.

Identification of the Diphtheria Bacillus—Allied Organisms

It is now recognised that the B. diphtheriæ is a member of a group of organisms with closely allied characters, which are of common occurrence and have a wide distribution as commensals of the skin and mucous membranes of man and the lower animals. This group corresponds with the genus Corynebacterium of the Association of American Bacteriologists, and its characters as modified by the Committee of the Medical Research Council may be stated as follows :

" Gram-positive rod-like forms, arranged usually in a palisade, not acid-fast, often with club-shaped swellings at the poles, generally with irregularly stained segments or granules, non-motile, without endospore formation, growing aerobically or under micro-aerophilic conditions, but often capable of anaerobic cultivation, never forming gas in carbohydrate media, in which they may or may not produce acidity ; they may or may not liquefy gelatin or serum."

Members of the genus Corynebacterium other than the diphtheria bacillus are usually known in this country as " diphtheroids." The term " pseudo-diphtheria " is sometimes used as synonymous with diphtheroid, though more frequently it is applied specially to Hofmann's bacillus (p. 482); it would be an advantage if the use of the term pseudo-diphtheria were discontinued. Diphtheroids have been obtained from the mouth, ear, nose, skin, genital organs, and even from the blood in certain diseases. They are to be met with in conditions of health, and they have been obtained from many diverse morbid conditions—from skin diseases, from coryza, from leprosy, from gun-shot wounds, and even from general paralysis of the insane. As has been found with other groups, the differentiation is a matter of considerable difficulty. Some are practically identical with the diphtheria bacillus both morphologically and culturally, and give the characteristic reaction with Neisser's stain ; others, again, differ in essential particulars. The fermentative action on sugars (p. 62) has also been called into requisition as a means of distinguishing them, but the results obtained cannot be said to be of a decisive character. The absence of the power of fermenting glucose or the presence of capacity to ferment saccharose may, however, be accepted in any particular case as sufficient to exclude the organism from being the diphtheria bacillus. Two species of

diphtheroids are known to produce pseudo-tuberculosis in sheep and mice respectively, but the diphtheroids obtained from the human body are not known to be responsible for any pathogenic effects. Further, cultures of these organisms are as a rule quite non-virulent when tested by inoculation of guinea-pigs. A few instances are recorded where diphtheroids have produced inflammatory and septicæmic conditions in these animals, but such an occurrence is exceptional. A diphtheroid organism corresponding fully with the diphtheria bacillus in its morphological, cultural, and fermentative characters cannot be distinguished from an avirulent diphtheria bacillus. We give below an account of the characters of two diphtheroids, namely, the xerosis bacillus and Hofmann's bacillus, as these are well recognised organisms of frequent occurrence.

From what has been said it will be clear that the scientific differentiation of the diphtheria organism may be a matter of great difficulty. With regard to the rules for practical guidance, however, there is general agreement as to the two following. In the first place, in cases of suspected diphtheria the obtaining of a bacillus in a serum culture from the throat, which has all the morphological and staining characters of the diphtheria bacillus, may be accepted as a positive result for all practical purposes. And, further, most will agree that a similar rule should hold in the first instance with regard to bacilli obtained from the throats of *immediate* contacts. In view, however, of the fact that diphtheria-like bacilli without virulence are present in the throats of some healthy individuals, and may also be present along with virulent bacilli in cases of diphtheria, it appears to us that no one should be regarded as a carrier, dangerous to the community, unless the organism in question is proved to possess virulence. Such a rule rests on the assumption that quite avirulent bacilli do not give rise to infection and may be disregarded. The results of the accumulated experience of numerous observers, however, support such a view. In the second place, a diphtheria-like bacillus obtained from another part of the body, with or without a lesion, should not be accepted as the diphtheria bacillus, however closely it resembles it, unless it is found on inoculation to produce the characteristic results.

Hofmann's Bacillus.—Pseudo-Diphtheria Bacillus (*Corynebacterium hofmanni*).—This organism, described by Hofmann in 1888, is probably the same as one observed by Löffler in the previous year, and regarded by him as being a distinct species from the diphtheria bacillus. The organism is a shorter bacillus than the diphtheria bacillus, with usually a single unstained septum running

across it, though sometimes there may be more than one (Fig. 126). The typical beaded appearance is rarely seen, and the characteristic reaction with Neisser's stain is not given, though in old cultures a few granules which stain deeply may sometimes be found. Involution forms may sometimes be produced by it. It grows readily on the same media as the diphtheria bacillus, but the colonies are whiter and more opaque. It does not form acid from glucose or other sugars, and is non-pathogenic to the guinea-pig. It is usually a relatively easy matter to distinguish this organism from the diphtheria bacillus.

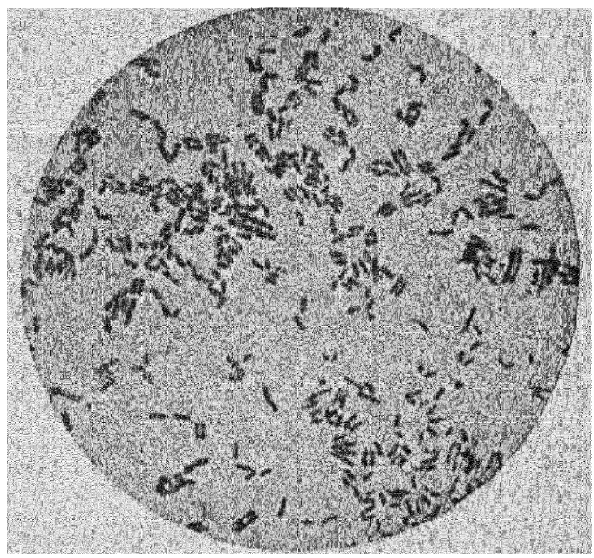


FIG. 126.—Pseudo-diphtheria bacillus (Hofmann's). Young agar culture. See also Plate III., Fig. 14.
Stained with thionin-blue. ×1000.

Hofmann's bacillus is of comparatively common occurrence in the throat in normal as well as diseased conditions, including diphtheria; it seems to be specially frequent in poorly nourished children of the lower classes. Cobbet found it 157 times in an examination of 692 persons, of whom 650 were not suffering from diphtheria. According to Boycott's statistics the time of its maximum seasonal prevalence precedes that of the diphtheria bacillus. Knowles, on the other hand, in India, found a greatly increased frequence of Hofmann's bacillus during an epidemic of diphtheria, whereas at other times it was relatively rare. It is still undetermined to what extent, if any, it is responsible for pathological changes in the throat. There is no evidence that Hofmann's bacillus can be transformed into a virulent B. diphtheriæ or *vice versa*, though this was at one time maintained by some.

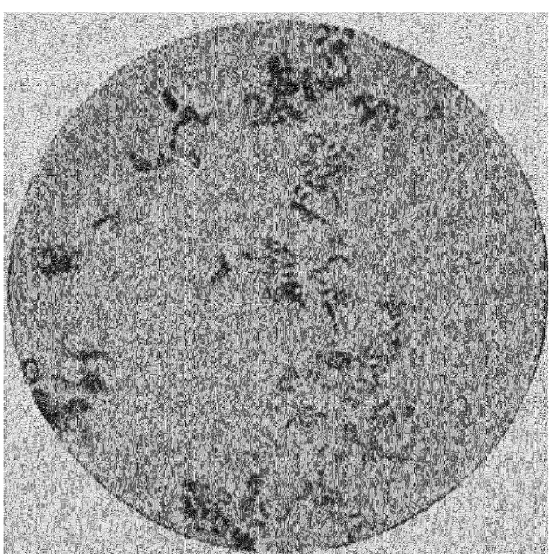


FIG. 127.—Xerosis bacillus from a young agar culture. ×1000.

Xerosis Bacillus (*Corynebacterium xerosis*).—This term has been given to an organism first observed by Kuschbert and Neisser in xerosis of the conjunctiva. It, however, has since been found in

many other affections of the conjunctiva and also in normal conditions; in fact, it is a usual commensal. Morphologically it is practically similar to the diphtheria bacillus, and even in cultures presents very minor differences; it, however, grows more slowly on serum, and its colonies are small and scaly, have a tough consistence, and a somewhat irregular margin. It is non-virulent to animals and produces acid from glucose and also from saccharose. The last property distinguishes it from the diphtheria bacillus. It is still doubtful whether it has any effect in the human subject, though it increases in numbers in abnormal states of the conjunctiva. Its morphological characters are shown in Fig. 127.

Action of the Diphtheria Bacillus—Summary.—From a study of the morbid changes in diphtheria and of the results produced experimentally by the bacillus and its toxins, the following summary may be given of its action in the body. Locally, the bacillus produces inflammatory change with fibrinous exudation, but at the same time cellular necrosis is also an outstanding feature. Though false membranes have not been produced by the toxins, a necrotic action may result when these are injected subcutaneously. The toxins also act upon the blood vessels, and hence œdema and tendency to hæmorrhage are produced; this action on the vessels is also exemplified by the general congestion of organs. The hyaline change in the walls of arterioles and capillaries, so often met with in diphtheria, is another example of the action of the toxin. The toxins have also a pernicious action on highly developed cells and on nerve fibres. Thus in the kidney cloudy swelling occurs, which may be followed by actual necrosis of the secreting cells, and along with these changes albuminuria is present. The action is also well seen in the case of the muscle fibres of the heart, which may undergo a sort of hyaline change, followed by granular disintegration and associated with leucocytic infiltration. These changes are of great importance in relation to heart failure in the disease. Changes of a somewhat similar nature have been observed in the nerve cells of the central nervous system, those lying near the capillaries, it is said, being affected first. There is also a striking change in the peripheral nerves, which is shown first by the disintegration of the medullary sheaths and to which paralysis is due.

Methods of Diagnosis.—These include: (*a*) *Microscopical Examination.*—For microscopical examination it is sufficient to tease out a piece of the membrane with forceps and rub it on a slide; if it be somewhat dry, a small drop of normal saline should be added. The films are then dried in the usual way, and stained with any ordinary basic stain, though methylene-blue is on the whole to be preferred, used either as a saturated watery solution or in the form of Löffler's solution. After staining for two or three

minutes, the films are washed in water and dried. No decolorising is necessary, as the blue does not over-stain. Neisser's stain (p. 109) may also be used with advantage, although it is to be noted that sometimes in a secretion the diphtheria bacillus does not react typically to this stain. Any secretion from the pharynx or other part is to be treated in the same way. Diagnosis by the microscopic examination is now little used, but it is sometimes justified in cases of urgency, though only in the hands of an experienced observer. In some cases the bacilli are present in characteristic form in such numbers as to leave no doubt in the matter.

(*b*) *Cultivation.*—For this purpose a piece of the membrane should be separated by forceps from the pharynx or other part when that is possible. It should be then washed well in a tube containing sterile water, most of the surface impurities being removed in this way. A fragment is then fixed in a platinum loop by means of sterile forceps, and a series of stroke cultures is made on the surface of Löffler's serum or similar medium, the same portion of the membrane being always brought into contact with the surface. More usually a swab taken from the membrane or the pharynx is smeared over the medium in a similar manner (no antiseptic solution should have been applied to the throat for some hours previously). The tubes are then incubated at 37° C., and are ready for examination in eighteen to twenty-four hours. A representative sample of the whole growth is obtained by rubbing a platinum loop over the surface; films are made from this, stained, and examined in the usual way, Neisser's stain being also applied.

The presence of organisms with the characteristic microscopic appearances and staining reactions in a case presenting the clinical features of diphtheria, may be taken as a positive result. In the case of an organism obtained from a suspected carrier or from lesions in parts other than the fauces or larynx, the organism ought to be isolated and further tests applied. Pure cultures may be obtained by means of successive strokes on serum medium, but the telluric acid medium will be found of service in the isolation. When the organism has been isolated its fermentative properties should be determined. If they correspond with those of the B. diphtheriæ (production of acid from glucose, no acid from saccharose), the virulence test must be applied; if they do not correspond it may be concluded that the organism is not a B. diphtheriæ.

(*c*) *Virulence Test.*—For this purpose the bacillus in question should be grown on a serum slant; the whole of the growth is then scraped off and suspended in saline and then injected subcutaneously into a guinea-pig. If the organism is the B. diphtheriæ the animal will die with the characteristic appearances (p. 471). The intracutaneous method (p. 472) may also be used, 0·15 c.c. of the suspension being injected. A portion of the skin of the guinea-pig may be depilated for this purpose by means of calcium sulphide. In cases where it is difficult to obtain a pure culture, what is called the "immediate" or "crude" virulence test may be applied. In this case the whole of an impure serum culture containing the organism may be injected subcutaneously, or a portion may be used by the intracutaneous method. As death may be produced by other organisms if present, care must be taken that the characteristic effects of the B. diphtheriæ are produced, and it is essential to use another animal treated with antitoxin as a control.

CHAPTER XVIII

EPIDEMIC INFLUENZA, INFECTIOUS CORYZA, WHOOPING-COUGH (PERTUSSIS)

Epidemic Influenza

The term "influenza" has been generally, and somewhat loosely, applied to various acute febrile conditions attended by catarrhal inflammation of the upper respiratory passages, and has thus come to refer not to a specific infectious disease but to conditions which may be etiologically different. Authentic European records over nearly two centuries show that at varying intervals there have occurred severe and widespread epidemics of a condition which, in the description of its clinical features, conforms to influenza. Such epidemics have shown a tendency to pandemic spread, and this is an outstanding epidemiological feature of the disease. It is therefore advisable to use the designation "epidemic or pandemic influenza" in referring to the disease in its widespread epidemic form, apart from the conditions so frequently diagnosed as influenza during non-epidemic intervals. Since the period of bacteriological investigation two such pandemics have occurred (1889–92 and 1918–21) and their etiology has been extensively studied. The disease exhibits an exceptionally marked degree of infectiousness and assumes a high incidence in any population in which it occurs, spreading with great rapidity. On the other hand, it quickly disappears or "burns out" in a community. In the 1889 and 1918 pandemics the outbreaks occurred in definite successive waves, and the duration of these pandemics thus extended over a considerable period before the condition ultimately became quiescent. The first accounts of the organism now known as the influenza bacillus were published simultaneously by Pfeiffer, Kitasato, and Canon, in January 1892. The two first-mentioned observers found it in the bronchial sputum, and obtained pure cultures, and Canon observed it in the blood in a few cases of the disease. This organism, up to the time of the recent pandemic of 1918, was generally accepted as the primary

causal agent, although absolute proof was wanting. As a result of the 1918 pandemic, the etiology of the disease was extensively restudied, and opinions have varied as to whether this organism represents the primary agent or only an associated and secondary infection. This question will be discussed later.

B. Influenzæ (Pfeiffer) (*Hæmophilus influenzæ*).—*Microscopical Characters.*—The influenza bacilli as seen in the sputum are very minute rods not usually exceeding 1·5 μ in length and 0·3 μ in thickness. They are straight, with rounded ends, and sometimes stain more deeply at the extremities (Fig. 128). The bacilli occur singly, in pairs, or form clumps by their aggregation. They take up the basic aniline stains somewhat feebly, and are best stained by a weak solution (1 : 10) of carbol-fuchsin applied for from five to ten minutes. They are Gram-negative. They are non-motile, and do not form spores. In the early stages of the more acute cases with catarrhal complications of the respiratory system, influenza bacilli are frequently present in large numbers and may be easily found. On the other hand, it is often difficult or impossible to find them, even when the symptoms are severe.

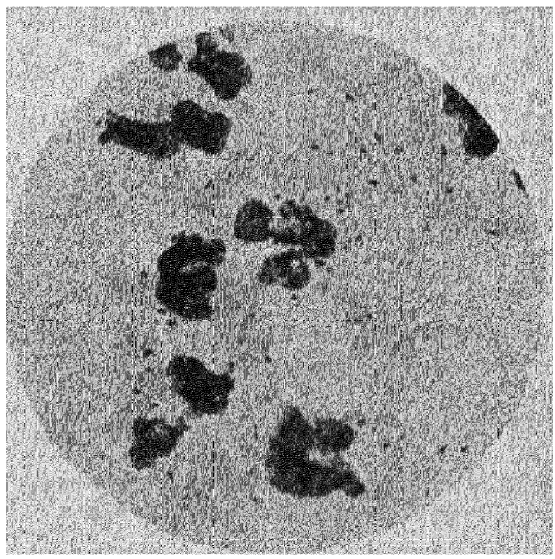


FIG. 128.—Film preparation of influenza sputum showing influenza bacilli along with leucocytes. ×1000.

Cultivation.—The best medium for the growth of the influenza bacillus is blood agar (see p. 54). Pfeiffer originally used blood-smeared agar for the cultivation of the organism. He obtained growths of the bacilli on agar which had been smeared with influenza sputum, but he failed to subculture them on ordinary agar media or on serum. The growth in the first cultures he considered to be probably due to the presence of certain organic substances in the sputum, and accordingly he tried the expedient of smearing the agar with drops of blood before making the inoculations. In this way he completely succeeded in attaining his object. The blood of various animals is suitable, as well as human blood ; and the favouring influence

of the blood would appear to be due to the hæmoglobin, as a solution of this substance is equally effective. Hence the B. influenzæ and closely allied organisms have been designated *hæmoglobinophilic*. The colonies of the influenza bacilli on blood agar, incubated at 37° C., appear within twenty-four hours, in the form of minute circular dots, almost transparent, like drops of dew (Fig. 129). When numerous, the colonies are scarcely visible to the naked eye, but when sparsely arranged they may reach the size of a pin's head. This size is generally reached on the second day. In cultures the bacilli may show considerable variations in size (Fig. 130) and in shape, and after a time involution forms (Fig. 131) may be present; they die out somewhat quickly, and in order to keep them alive sub-

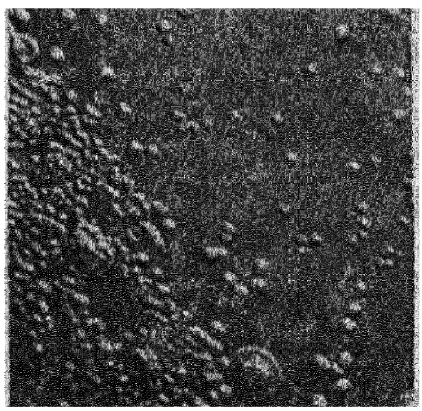


FIG. 129.—Colonies of influenza bacilli on blood-agar plate. (There are a few larger colonies of other organisms.) ×5.

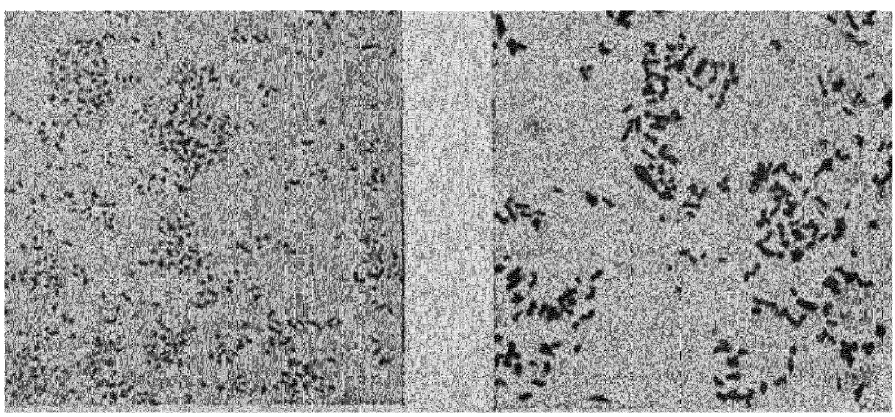


FIG. 130.—Film preparation from young culture of influenza bacillus. Stained with weak carbol-fuchsin. ×1000.

FIG. 131.—Film preparation from older culture of influenza bacillus, showing involution forms. ×1000.

cultures should be made every four or five days until the organism is accustomed to culture medium. Even on subculture, growth on the ordinary agar media is practically absent; growth may occur, however, when other organisms are present

—that is, it is favoured by symbiosis. Neisser, for example, was able to cultivate the influenza bacillus on plain agar through several generations by growing the xerosis bacillus along with it ; dead cultures of the latter had not the same favouring effect. It has also been noted that the growth of the influenza bacillus is aided by the concomitant growth of pneumococci and staphylococci. Growth occurs in blood bouillon and forms a thin whitish deposit at the bottom of the tube or flask. The limits of growth are from 25° to 42° C., the optimum temperature being that of the body. The influenza bacillus is a strictly aerobic organism. Owing to its peculiar hæmoglobinophilic character the B. influenzæ has been carefully studied in recent years as regards its requirements for growth in artificial culture, and interesting data have been obtained. Apparently this organism will only flourish in the presence of two growth-promoting principles. One of these, designated the X factor (Avery), is a highly thermostable substance which remains unaltered even after autoclaving (*e.g.* at 120° C.). This principle is associated with hæmoglobin and exhibits a peroxidase action. Some workers regard the principle as hæmatin. The other factor (V) is less thermostable, being destroyed at temperatures above 100° C., and appears to be of the nature of a vitamin. Blood agar contains both these growth-stimulating substances. Some vegetable tissues, *e.g.* potato, also supply them. Thus in the case of potato the stimulating action, according to Fildes, is due to the peroxidase present acting like hæmoglobin in accelerating the transfer of atmospheric oxygen to the organism. Thjøtta has shown that the V factor is contained in red cells, bacteria, yeasts, and vegetables. It is possible that this principle is identical with the water soluble vitamin B. Certain artificial media have proved specially valuable for the isolation and growth of the bacillus. The oleate-blood-agar of Avery[1] favours the growth of B. influenzæ and inhibits that of certain other organisms frequently present in sputum, *e.g.* streptococci and pneumococci. It is therefore particularly applicable for isolating the organism. The so-called " chocolate " agar, prepared by adding blood to nutrient agar at a temperature of 90° C., yields an abundant growth of B. influenzæ and is used for maintaining laboratory cultures which are somewhat more luxuriant on this medium than on agar containing unaltered

[1] 5 c.c. of a sterilised 2 per cent. solution in water of sodium oleate and 1 c.c. of a sterile suspension of rabbit's blood cells (prepared by centrifuging defibrinated blood, removing the serum, and making up to original volume with sterile bouillon) are added to 94 c.c. of melted nutrient agar at 90° C. The agar should have a P_H of 7 to 7·4.

blood. It has been supposed that the difference between the altered and unaltered blood depends on the fact that the fresh blood deviates oxygen from the bacillus in virtue of its greater oxygen affinity (Fildes). A considerable proportion of strains of B. influenzæ have been found to produce indol in culture; others lack this property. Strains of B. influenzæ constitute a serologically heterogeneous group and represent a multiplicity of races. According to Jordan and Sharp, and others, strains may even exhibit serological individuality. This is analogous to what has been observed in the B. coli group (*vide* p. 401).

The powers of resistance of this organism are of a low order. Pfeiffer found that dried cultures kept at the ordinary temperature were usually dead in twenty hours, and that if sputum were kept in a dry condition for two days cultures could be no longer obtained. From these experiments it follows that outside the body in ordinary conditions they can remain alive only for a short time.

Distribution in the Body.—The bacilli are found chiefly in the respiratory passages in influenza. They may be present in large numbers in the nasal secretion, generally mixed with a considerable number of other organisms, but it is in the small masses of greenish-yellow sputum from the bronchi that they are present in largest numbers, in many cases almost in a state of purity. They occur in clumps which may contain as many as 100 bacilli, and in the early stages of the disease are chiefly lying free. As the disease advances, they may be found in considerable numbers within the leucocytes, and towards the end of the disease a large proportion have this position. They may persist for weeks after symptoms of the disease have disappeared, and may still be detected in the sputum. Especially is this the case when there is any chronic pulmonary disease. They occur also in large numbers in the capillary bronchitis and catarrhal pneumonia of influenza, as Pfeiffer showed by means of sections of the affected parts. In these sections he found the bacilli lying amongst the leucocytes which filled the minute bronchi, and also penetrating between the epithelial cells and into the superficial parts of the mucous membrane. M'Intosh has shown that at a very early stage the bacilli are present in the upper respiratory tract but absent from the lung. Other organisms also, *e.g.* streptococci, pneumococci, may be concerned in the pneumonic conditions following influenza. Occasionally in the foci of suppurative softening in the lung the influenza bacilli have been found in a practically pure condition. In cases of empyema the organisms present would appear to be

chiefly streptococci and pneumococci, but influenza bacilli may also be present; whilst in the gangrenous conditions, which sometimes occur, a great variety of organisms has been found.

Pfeiffer's observations on a large series of cases convinced him that the organism was very rarely present in the blood—that in fact its occurrence there must be looked upon as exceptional. The conclusions of other observers have, on the whole, confirmed this statement. The bacillus may be present in other lesions complicating influenza. Pfeiffer found it in inflammation of the middle ear, and it has been found in meningitis following influenza. Pfuhl considered that in these cases the path of infection is usually a direct one through the roof of the nasal cavity. This observer also found *post mortem*, in a rapidly fatal case with profound general symptoms, influenza bacilli in various organs, both within and outside of the vessels. In a few cases also the bacilli have been found in the brain and its membranes, with little tissue change in the parts around.

Extensive observations on the bacteriology of the respiratory system show that "influenza-like" bacilli may be present in a great variety of conditions; and though these may represent a biological group possessing somewhat different degrees of pathogenicity, it is impossible to differentiate them from strains isolated from influenza. These bacilli have been obtained from the fauces, bronchi, and lungs in various inflammatory conditions, and also in specific fevers. They are not infrequent in phthisis, in bronchiectatic cavities, and in chronic inflammation of the nasal sinuses. They may also be recovered frequently from the nose and throat in quite healthy persons. All these organisms are very restricted in their growth, and like Pfeiffer's bacillus, are hæmoglobinophilic. Some of them appear a little larger than the influenza bacillus, and tend to form short filaments, but others are quite indistinguishable. Most of them also seem to have very feeble pathogenic properties towards the lower animals. It can scarcely be claimed as possible to distinguish Pfeiffer's bacillus from those allied types by biological characters. A hæmoglobinophilic organism has been not infrequently found in cases of acute primary meningitis occurring in young subjects (p. 296). In the cerebro-spinal fluid this organism may appear as somewhat elongated filaments, and it was originally thought to be of the nature of a leptothrix. It is, however, closely allied biologically to the influenza bacillus, and the filamentous form is not a constant character. This organism differs from typical strains of B. influenzæ in its virulence for rabbits (Rivers). Hæmolytic hæmoglobinophilic bacilli similar in growth requirements to B. influenzæ have been described (Rivers).

Experimental Inoculation.—There is no evidence that any of the lower animals suffer from influenza under natural conditions, and accordingly we cannot look for very definite results

from experimental inoculation. Pfeiffer, by injecting living cultures of the organism into the lungs of monkeys, in three cases produced a condition of fever of a remittent type. There was, however, little evidence that the bacilli had undergone multiplication, the symptoms being apparently produced by their toxins. He accordingly came to the conclusion that the influenza bacilli contain toxic substances which can produce in animals some of the symptoms of the disease, but that animals are not liable to infection, the bacilli not having the power of multiplying to any extent in their tissues. In the case of rabbits, intravenous injection of living cultures produces dyspnœa, muscular weakness, and slight rise of temperature; death may follow. Wollstein distinguished virulent and avirulent types according to the result on intravenous injection in the rabbit; the virulent types caused death in about twenty-four hours, the bacilli being numerous in the blood. The dose used, however, was comparatively large, namely, a blood-agar culture for a rabbit of 1000 grams. Strains from the respiratory tract were non-virulent by this test; those from the blood and meninges, and rarely from pneumonic lung; were virulent. Wollstein found that a fatal cerebro-spinal meningitis could be produced in monkeys by the subdural injection of virulent cultures. M'Intosh has claimed that B. influenzæ produces a soluble toxin, and that inoculation of animals, *e.g.* rabbits and guinea-pigs, with filtered cultures, leads to pathological changes in the lung which show a close resemblance to those observed in man. Blake and Cecil produced an influenza-like illness in monkeys by inoculation with B. influenzæ cultures. Cecil and Steffen, by introducing virulent influenza bacilli into the throat and nose in the human subject, produced an acute respiratory disease which, however, fell short of typical influenza.

Reviewing the extensive experimental work that has now been carried out with B. influenzæ, we may say that conclusive proof of its primary etiological relationship to the disease has not been obtained. The frequent association of the organism with the epidemic disease has been well established, but it is not invariably present. Its occurrence is, moreover, specially related to the catarrhal complications. During the 1918 pandemic, cases were met with in which the fever and general manifestations were exceedingly marked but unaccompanied by obvious inflammatory involvement of the respiratory tract, and B. influenzæ was not detectable in the nose and throat secretions. Even in cases with pulmonary complications, the organism could not be found constantly in the sputum when

examined by the best methods. Such observations led to the view that, contrary to previous belief, this organism is not the primary agent in epidemic influenza, though it was admitted that it represents a frequent concomitant infection and may be responsible, like streptococci and pneumococci, for pulmonary complications. It has been argued, however, by many observers, that Pfeiffer's bacillus is the specific causal agent, and while its occurrence in catarrhal conditions of the respiratory tract apart from epidemic influenza has been recognised, the epidemic disease has been regarded as due to a periodic exaltation in virulence. M'Leod, Ritchie, and Dottridge have claimed that the comparative incidence of infection with B. influenzæ before, during, and after the 1918 epidemic, is in favour of this organism being the cause, and they suggest that its virulence is progressively increased by " passage " in the smaller epidemics during the years prior to the major epidemic. In the recent pandemic, preventive inoculation with vaccines of B. influenzæ, along with other associated organisms, *e.g.* pneumococci, streptococci, was extensively applied. There has been no undoubted statistical proof of the efficacy of the procedure, and evidence from this source as to the etiological relationship of B. influenzæ to the epidemic disease is awanting. The observed relationships of the organism to lesions in the lungs and elsewhere leave no room for doubt that it is possessed of pathogenic properties, but its causal relationship to epidemic influenza has not been established.

Methods of Examination.—(*a*) *Microscopic.*—A portion of the greenish-yellow purulent material which often occurs in little round masses in the sputum should be selected, and film preparations should be made in the usual way. Films are best stained by dilute carbol-fuchsin (1 : 10), staining being prolonged for ten minutes at least. In sections of the tissues, such as the lungs, the bacilli are best brought out, as shown by Pfeiffer, by staining with the same solution as above for half an hour. The sections are then placed in alcohol containing a few drops of acetic acid, in which they are dehydrated and slightly decolorised at the same time. They should be allowed to remain till they have a moderately light colour, the time varying according to their appearance. They are then washed in pure alcohol, cleared in xylol, and afterwards mounted in balsam.

(*b*) *Cultures.*—A suitable portion of the greenish-yellow material having been selected from the sputum, it should be washed well in several changes of sterilised water. A loopful should then be used to make successive strokes on the surface of blood-agar plates. The oleate-blood-agar medium of Avery (*vide supra*) in virtue of its selective action is specially useful for isolating the organism. The plates should be incubated at 37° C., when the transparent colonies

of the influenza bacillus will appear, usually within twenty-four hours. These should fail to grow in subculture on ordinary agar media.

The Question of a Filterable Virus as the Cause of Epidemic Influenza—Bacterium Pneumosintes

The inconstancy of Pfeiffer's bacillus in epidemic influenza, the fact that indistinguishable organisms may occur in various inflammatory conditions of the respiratory system, and the absence of experimental proof of its etiological relationship to the disease, led to further inquiry being made into the etiology of influenza during the pandemic of 1918 and the following years. The knowledge that had been obtained regarding the causation of certain specific infectious diseases by filter-passing viruses directed attention particularly to the question of whether this epidemic disease was the result of infection by such a virus. Further, the extreme infectiousness of the condition, by analogy with other conditions known to be due to filterable viruses, seemed to favour this view of the etiology of the disease. In the early stages of the pandemic, various workers claimed to have established the existence of a filter-passer in the disease (Gibson, Bowman, and Connor; Bradford, Bashford, and Wilson; Nicolle and Lebailly; Yamanouchi, Sakami, and Iwashima; and others). Certain of these claims, however, were subject to serious criticism in regard to the technique of the experimental work on which they were based, though the Japanese workers were able to produce an influenza-like disease in susceptible persons by the injection into the nose and throat of filtrates of sputum and blood from influenza cases.

In 1921 Olitsky and Gates described a filter-passing virus in the naso-pharynx of cases of influenza which was not an ordinary bacterium. Inoculated intratracheally in rabbits, it produced fever, leucopenia, hæmorrhages in the lung with œdema and emphysema. This agent could only be demonstrated in the earliest stage of the illness and was absent after thirty-six hours from the onset. It could not be isolated from other conditions or from healthy persons. When inoculated into rabbits, it apparently underwent increase in the lungs and could be passed through animals in series. This agent, derived from the naso-pharyngeal secretions and the lungs of experimental rabbits, could be passed through Berkefeld V and N filters, and like certain other filterable viruses withstood 50 per cent. glycerol for long periods. In subsequent studies of the virus, Olitsky and Gates identified it as an exceedingly minute

bacilloid structure measuring 0·15 to 0·3 μ in length which they designated *Bacterium pneumosintes* (*Dialister pneumosintes*). Cultures from the filtrates were obtained in Smith-Noguchi medium (*vide* p. 569), and, when inoculated intratracheally in rabbits, reproduced the effects already described as following the inoculation of filtrates of the naso-pharyngeal secretion. Olitsky and Gates regarded the condition produced in animals by their filterable organism as analogous to the primary condition in epidemic influenza, and supposed that this is the causal organism which predisposes the lung tissue to secondary microbic invasion, with all the characteristic complications of the disease. Bacterium pneumosintes undoubtedly represents a definite biological entity, and the occurrence of a similar organism in influenza has been confirmed by others in different parts of the world (Gordon, Lister, and others).

While the characteristic morphological appearances are those of a very small coccal or bacillary organism of the dimensions given above, it has been found that after continuous artificial culture the bacillary form becomes more marked, the length of the organism increasing to 0·5 μ. It may occur singly, in pairs, or even in short chains. It is best demonstrated in culture by staining with polychrome methylene-blue. It can also be stained, however, with simple stains, and is Gram-negative. It is a strict anaerobe, and when first grown in Smith-Noguchi medium a cloudiness forms in the medium, starting after three to four days at 37° C., at the foot of the tube round the tissue fragment. Colony growths can be obtained under anaerobic conditions on blood agar. The colonies are very small and transparent and take several days to appear. In more recent studies, Olitsky and Gates have shown that other anaerobic filter-passing Gram-negative organisms biologically allied to B. pneumosintes may occur in the throat secretions, which can be differentiated serologically from the latter, and they claim that this organism is specifically associated with influenza. The claims put forward by these workers have tended towards the elucidation of the etiological problem of epidemic influenza, and taking all the facts into consideration, it seems reasonable to believe that epidemic influenza is primarily a specific infection of the respiratory system due to a filter-passing virus which predisposes the mucous membrane of the respiratory tract and the pulmonary tissue to secondary infection by B. influenzæ, the pneumococcus, streptococci, etc. But it cannot be said that the etiological rôle of B. pneumosintes has yet been established beyond doubt.

Infectious Coryza (the "Common Cold")

The infectious nature of this prevalent condition is now well recognised, and the infection can frequently be traced to contact with other cases of the same condition, the incubation period being within three to four days. The condition is characterised by a preliminary dryness of the nasal mucosa, often with sneezing, followed by an abundant watery discharge from the nostrils; there may be some degree of malaise, headache, and, in some cases, slight fever. After two or three days the discharge becomes less, and tends to assume a purulent character; restoration to normal then occurs after a varying number of days. It is to be noted that in the purulent stage, secondary spread may occur, *e.g.* to the nasal sinuses, middle ear, and at this stage various pyogenic organisms commonly found in inflammatory conditions of the upper respiratory passages may be present, *e.g.* staphylococci, streptococci, pneumococci, M. catarrhalis, etc.

In 1914, Kruse claimed that the condition was due primarily to a filterable virus, and he was able to reproduce the condition in volunteers after an incubation period of one to four days, by introducing into the nose the filtered nasal secretion from a case. The filtrate, obtained by means of a Berkefeld filter, was apparently free from any recognisable organism.

In 1915–16 Foster claimed to have confirmed these results, and also to have cultivated in the Smith-Noguchi medium an anaerobic organism similar to the "globoid bodies" described by Flexner and Noguchi in epidemic poliomyelitis.

Olitsky and McCartney have more recently repeated this work, using as the inoculum naso-pharyngeal secretions from early cases in the first three to eighteen hours of the disease, the material being filtered through Berkefeld V and N filters. They succeeded in transmitting the condition to volunteers, and further, were able to pass it from an experimentally infected case. The incubation period was from eight to twenty-four hours. The heated material was inactive and transmission did not occur as the result of inoculation with filtered secretions from cases eighteen to twenty hours after the onset of symptoms. Intratracheal injection in rabbits both with filtered and unfiltered naso-pharyngeal washings was without effect, contrasting in this respect with the same experiment carried out with the nasal washings from early cases of influenza (*vide* p. 494). These observers were unable to isolate by culture in the Smith-Noguchi medium, and on anaerobic blood-agar plates, any specific cultivable organism.

From these observations it would appear probable that the common infectious "cold" is primarily due to a transmissible filterable virus, which may predispose to secondary infection with pyogenic bacteria.

Whooping-Cough

Up to the year 1906, the chief result of bacteriological observations, of which those of Spengler, Krause and Jochmann, and Davis may be mentioned, had been to demonstrate the very frequent presence of minute influenza-like and hæmoglobinophilic bacilli in the sputum and also in the lesions in this disease. In the year mentioned, however, Bordet and Gengou published an account of another minute organism, designated B. pertussis, and brought forward certain facts which gave strong support to its etiological relationship.

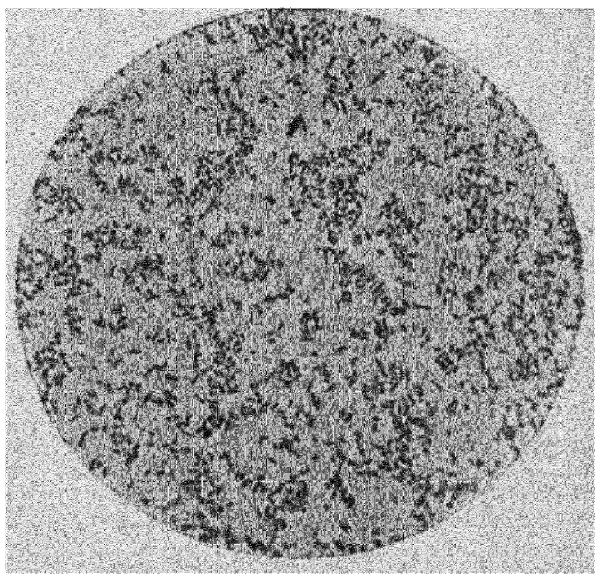


Fig. 132.[1]—Film preparation from a twenty-four hours' culture of the bacillus of whooping-cough. (Bordet-Gengou.) Stained with dilute carbol-fuchsin. ×1000.

Characters of the Bacillus Pertussis.—(*Hæmophilus pertussis*).—The organism, as seen, for example, in the sputum, occurs in the form of minute oval rods scarcely larger than the influenza bacillus. They stain rather faintly with ordinary stains, and their margin and extremities are often more deeply coloured than the centre, which may appear as an uncoloured spot; they are Gram-negative and do not form spores. In cultures they present the same characters, and are less pleomorphous than the influenza bacillus (Fig. 132). They are specially numerous at the beginning of the disease, and they may be found in large numbers in almost pure culture in the opaque whitish sputum expectorated from the bronchi; as the disease advances they become scanty, and may disappear when the symptoms of the

[1] We are indebted to Dr. Bordet for the culture from which this preparation was made.

disease are still prominent. The bacillus has not been found in the blood, unless as an agonal phenomenon (Klimenko). Bordet and Gengou succeeded in obtaining pure cultures on the glycerin-potato-blood-agar medium described on p. 56, and this was found to be the most suitable of all the media tried. It has been recommended that this medium should be standardised to a P_H 6·6, *i.e.* more acid than the usual reaction (Povitzky). In the first cultures, growth is very scanty and may be almost invisible, consisting of minute transparent colonies, but later it becomes much more abundant, and subcultures may also be readily made on ordinary blood- or serum-agar media. As compared with that of the influenza bacillus, growth after several generations is thicker and less transparent and the margins are more sharply marked off; it also presents a tenacious character. The presence of hæmoglobin, though favouring the growth, is not so essential as in the case of the B. influenzæ. The organism is a strict aerobe, and in the case of cultures in fluid media, *e.g.* serum bouillon, the tubes ought to be placed in a sloped position, in order to expose a large surface to the air. Bordet and Gengou completely confirmed the observations mentioned above as to the very frequent, almost constant, presence of influenza-like bacilli. They obtained growths of these organisms, and on comparing them with their own bacillus found that distinct cultural differences could be made out. The most marked distinctions were, however, obtained on studying the serum reactions of convalescents from the disease. They found that in many cases, though not invariably, such sera agglutinated their bacillus, but none of the influenza-like organisms. But the most important result was that in every case examined, the serum of convalescents gave the fixation of complement reaction very markedly with the whooping-cough bacillus, but with none of the others. This means, of course, that a specific antibody to the bacillus (immune-body) was present in the serum, and points to a true infection with the organism (p. 134). The results of the application of the test to adults suffering from bronchial irritation have been to show that they more frequently suffer from the infection than was formerly supposed, the paroxysmal stage being often absent.

Pathogenic Effects.—The general results obtained by Bordet and Gengou were that the ordinarily used animals were not susceptible to true infection with the bacillus, but that it contained a powerfully acting endotoxin, which produced both local and general effects. The injection of a small quantity of

the bacillus into the eye of a rabbit produced a local necrosis, with little inflammatory change, and the introduction of dead, as well as living, cultures into guinea-pigs caused death from toxic action, there being hæmorrhagic œdema locally, and hæmorrhages and necrotic foci in organs. Similar results were obtained with an endotoxin prepared according to Besredka's method. They advanced the view that the bacillus is present in large numbers at the beginning of the disease, and inflicts some local damage on the bronchial tubes which may persist after the disappearance of the bacillus and keep up the irritation. It was not found possible to obtain an antitoxin to this toxin. Very important results were, however, obtained by Klimenko, who succeeded in infecting monkeys and young dogs by intratracheal injection of pure cultures of the bacillus. After a period of incubation, there occurred an illness in which symptoms of pulmonary irritation and irregular pyrexia were outstanding features. Usually, in the case of the dogs, a fatal result followed after two or three weeks, and *post mortem* there were found catarrhal changes in the respiratory tract and sometimes patches of broncho-pneumonia, from which the bacillus could be recovered in pure culture. The serum of the infected animals gave the fixation of complement reaction. A specially interesting fact is that a number of healthy young dogs contracted the disease by contact with the inoculated. Fraenkel also obtained positive results, closely similar to those of Klimenko, on inoculation with pure cultures of the bacillus.

The results of Bordet and Gengou have received general confirmation, although it is to be noted that Fraenkel and also Wollstein failed to obtain the fixation of complement reaction with the serum of convalescents. This discrepancy may depend on the differences in the methods used by the various observers. It is impossible to make a definite pronouncement on the subject. We can only say that there is strong evidence for the etiological relationship of Bordet and Gengou's bacillus, and that their observations have been confirmed by others.

Methods of Examination.—A portion of sputum expectorated during a paroxysm of coughing should be obtained at as early as possible a stage of the disease; film preparations should be made in the usual way and stained by Gram's method and by carbol-thionin-blue. If the characteristic bacilli largely preponderate, tubes or plates of the Bordet-Gengou medium may then be inoculated and incubated. If numerous colonies of other organisms develop, a portion of the intervening agar should be scraped with a needle and fresh tubes inoculated. As already said, growth is at first very scanty but becomes more luxuriant in subcultures.

B. influenzæ and organisms of this type which may occur in sputum require careful differentiation from B. pertussis. The strictly hæmoglobinophilic character of B. influenzæ and the features of the growth of B. pertussis after several subcultures, as compared with that of the former (*vide supra*), serve to distinguish the two organisms. While B. pertussis may be grown on a serum medium and even on an ordinary nutrient agar, B. influenzæ completely fails to develop on such media.

CHAPTER XIX

PLAGUE AND TULARÆMIA

PLAGUE

THE bacillus of Oriental plague or bubonic pest was discovered independently by Kitasato and by Yersin during the epidemic

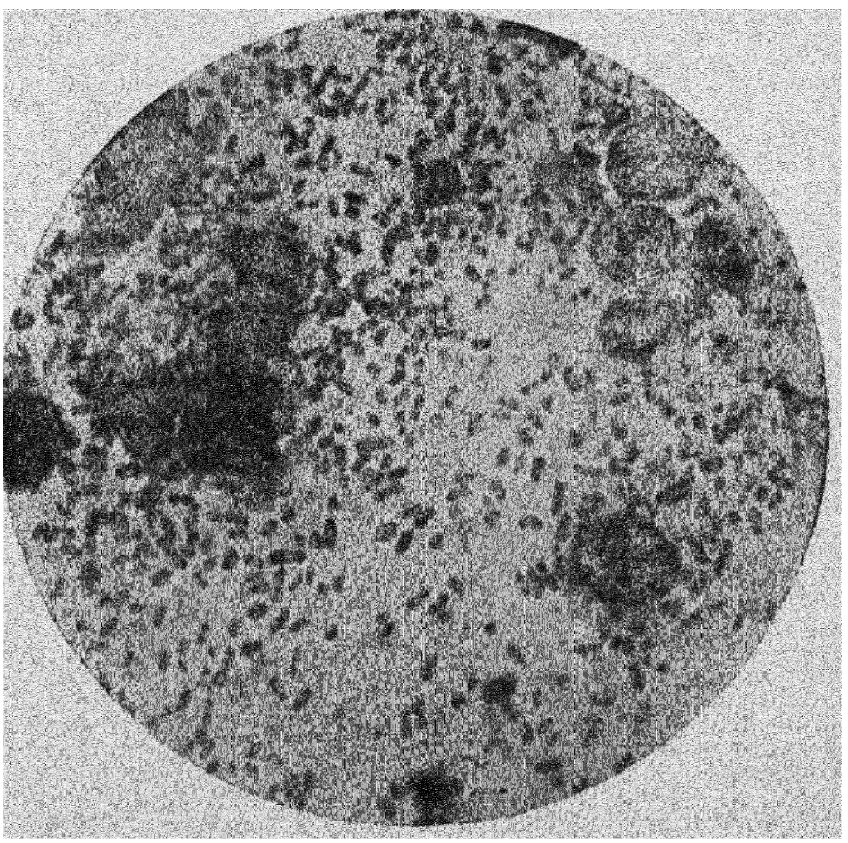


FIG. 133.—Film preparation from a plague bubo, showing enormous numbers of bacilli, most of which show well-marked bipolar staining.
Stained with weak gentian-violet. ×1000.

at Hong-Kong in 1894. They cultivated the organism from a large number of cases of plague, and reproduced the disease in susceptible animals by inoculation of pure cultures. It is to

be noted that during an epidemic of plague, sometimes even preceding it, a high mortality has been observed amongst certain wild rodents, especially rats and mice, and that from the bodies of these animals found dead in the plague-stricken district, the same bacillus was obtained by Kitasato and also by Yersin. Thus it is now well established that plague is an epizootic disease of rats and various other rodents in certain parts of the world, and that the human disease originates from an animal source.

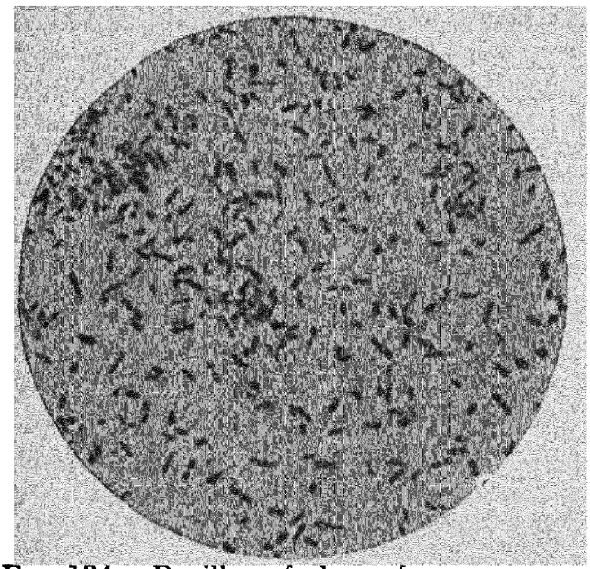


FIG. 134.—Bacillus of plague from a young culture on agar.
Stained with weak carbol-fuchsin. ×1000.

Bacillus of Plague (*Pasteurella pestis*).—*Microscopical Characters.*—As seen in the affected glands or buboes in this disease, the bacilli are small oval rods, somewhat shorter than the typhoid bacillus, and about the same thickness (Fig. 133), though considerable variations in size occur. They have rounded ends, and in stained preparations a portion in the middle of the bacillus is often left uncoloured, giving the so-called "polar staining." In films from the tissues they are found scattered amongst the cells, for the most part lying singly, though pairs are also seen. On the other hand, in cultures in fluids, *e.g.* bouillon, they grow chiefly in chains, sometimes of considerable length, the form known as strepto-

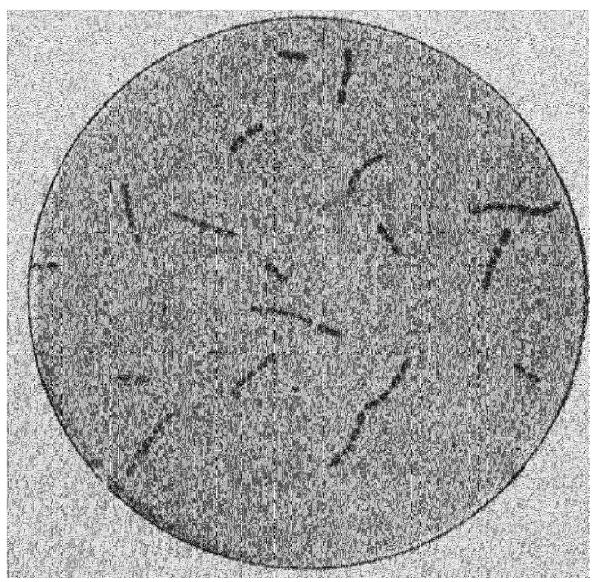


FIG. 135.—Bacillus of plague in chains showing polar staining. From a young culture in bouillon.
Stained with thionin-blue. ×1000.

bacillus resulting (Fig. 135). In young agar cultures the bacilli show greater variation in size, and polar staining is less marked than in the tissues (Fig. 134); sometimes forms of considerable length are present. After a time involution forms appear, especially when the surface of the agar is dry; but the formation of these is much more rapid and more marked when 2 to 5 per cent. of sodium chloride is added to the medium, constituting the so-called "salt agar" (Hankin and Leumann). On this medium the involution forms assume a great size and a striking variety of shapes, large globular, oval, or pyriform bodies resulting (Fig. 136). Sometimes in the tissues they are seen to be surrounded by an unstained capsule, though this appearance is by no means common. They do not form spores, and are non-motile. They stain readily with the basic aniline stains, but are Gram-negative.

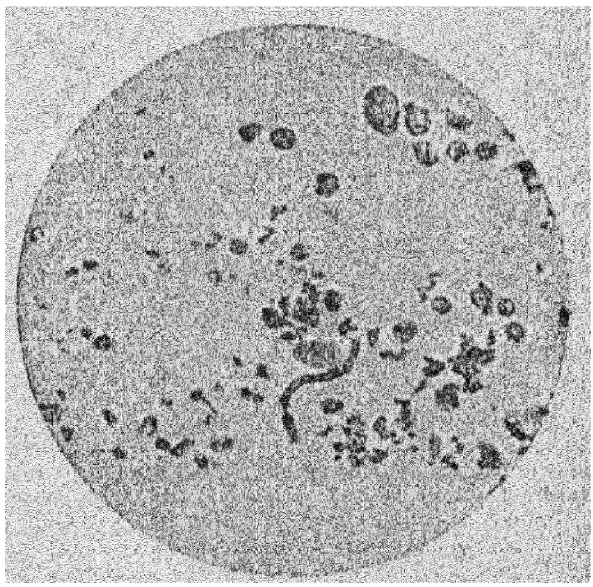


FIG. 136.—Culture of the bacillus of plague on 4 per cent. salt agar, showing involution forms of great variety of size and shape. See also Plate IV., Fig. 17. Stained with carbol-thionin-blue. ×1000.

Cultivation.—From the affected glands, etc., the bacillus can readily be cultivated on the ordinary media. It grows well at the temperature of the body, though its optimum is somewhat lower, about 30° C., and growth occurs even below 18° C. On *agar* and on *blood serum* the colonies are whitish circular discs of somewhat transparent appearance, and with a smooth, shining surface. When examined with a lens, their borders appear slightly wavy. In stroke cultures on agar there forms a continuous line of growth with the same appearance, showing partly separated colonies at its margins. When agar cultures are kept at the room temperature, some of the colonies may show a more luxuriant growth with more opaque appearance than the rest of the growth, the appearance in fact being often such as to suggest the presence of impurities in the cultures. In stab cultures in *peptone gelatin*, growth takes place along the needle track as a white line, composed of small spherical colonies. On the

surface of the gelatin a thin, semi-transparent layer may form, which is usually restricted to the region of puncture, though sometimes it may spread to the wall of the tube; sometimes, however, there is practically no surface growth. There is no liquefaction of the medium. In *bouillon* the growth usually forms a slightly granular or powdery deposit at the foot and sides of the flask, somewhat resembling that of a streptococcus. If oil or melted butter is added to the bouillon so that drops float on the surface, then a striking mode of growth may result, to which the term "stalactite" has been applied. This consists in the growth starting from the under surface of the fat globules and extending downwards in the form of pendulous, string-like masses. These masses are exceedingly delicate, and readily break off on the slightest shaking of the flask; accordingly during their formation the culture must be kept absolutely at rest. This manner of growth constitutes an important but not absolutely specific character of the organism; unfortunately it is not supplied by all strains of the organism, and varies from time to time with the same strain. The organism flourishes best in an abundant supply of oxygen; in strictly anaerobic conditions almost no growth takes place. The B. pestis ferments glucose and mannite without gas production, but has no such action on lactose, saccharose, or dulcite.

The organism in its powers of resistance corresponds with other spore-free bacilli, and is readily killed by heat, an exposure for an hour at 58° C. being fatal. On the other hand, it has remarkable powers of resistance against cold; it has been exposed to a temperature several degrees below freezing-point without being killed. Experiments on the effects of drying have given somewhat diverse results, but as a rule the organism has been found to be dead after being dried in air for two or three days, though sometimes it has survived the process for a longer period; exposure to direct sunlight for three or four hours kills it. The general result has been to show that the organism does not remain alive in natural conditions for long outside the animal body.

Anatomical Changes and Distribution of Bacilli.—The disease occurs in several forms, the *bubonic* and the *pulmonary* being the best recognised; to these may be added the *septicæmic*. The most striking feature in the *bubonic* form is the affection of the lymphatic glands, which undergo intense inflammatory swelling, attended with hæmorrhage, and generally ending in a greater or less degree of necrotic softening if the patient

lives long enough. The connective tissue around the glands is similarly affected. The bubo is thus usually formed by a collection of enlarged glands fused by the inflammatory swelling. True suppuration is rare. Usually one group of glands is affected first, constituting the primary bubo—in the great majority the inguinal or the axillary glands—and afterwards other groups may become involved, though to a much less



FIG. 137.—Section of a human lymphatic gland in plague, showing the injection of the lymph paths and sinuses with masses of plague bacilli—seen as black areas.
Stained with carbol-thionin-blue. ×50.

extent. Along with these changes there is great swelling of the spleen, and often intense cloudy swelling of the kidneys, liver, and other organs. There may also occur secondary areas of hæmorrhage and necrosis, chiefly in the lungs, liver, and spleen, also occasionally in the skin. The bacilli occur in enormous numbers in the swollen glands, being often so numerous that a film preparation made from a scraping almost resembles a pure culture (Fig. 133). In sections of the glands in

the earlier stages, the bacilli are found to form dense masses in the lymph paths and sinuses (Fig. 137), often forming an injection of them; they may also be seen growing as a fine reticulum between the cells of the lymphoid tissue. At a later period, when disorganisation of the gland has occurred, they become irregularly mixed with the cellular elements. Later still they gradually disappear, and when necrosis is well advanced it may be impossible to find any—a point of importance in connection with diagnosis. In the spleen they may be very numerous or they may be scanty, according to the amount of blood infection which has occurred; in the secondary lesions mentioned they are often abundant. In the *pulmonary* form the lesion is the well-recognised "plague pneumonia." This is of broncho-pneumonic type, though large areas may be formed by confluence of the consolidated patches, and the inflammatory process is usually attended by much hæmorrhage; the bronchial glands show inflammatory swelling. Clinically there is usually a fairly abundant frothy sputum often tinted with blood, and in it the bacilli may be found in large numbers. Sometimes, however, cough and expectoration may be absent. The disease in this form is almost invariably fatal; it is also extremely infective. In the *septicæmic* form proper there is no primary bubo discoverable, though there is almost always slight general enlargement of lymphatic glands; here also the disease is of specially grave character. A bubonic case may, however, terminate with sèpticæmia; in fact, all intermediate forms occur. In the various forms of the disease the bacilli occur also in the blood, in which they may be occasionally found during life by microscopic examination, chiefly, however, just before death in very severe and rapidly fatal cases. The examination of the blood by means of cultivation experiments is, however, a much more reliable procedure. For this purpose about 5 c.c. of blood may be withdrawn from a vein and distributed in flasks of bouillon (p. 146). It may be said from the results of different investigators that the bacillus may be obtained by culture in fully 50 per cent. of the cases, though the number will necessarily vary in different epidemics. The Advisory Committee, appointed by the Secretary of State for India in 1905, found that in some septicæmic cases the bacilli may be present in the blood in large numbers, two, or even three, days before death, though this is exceptional.

The above types of the disease are usually classified together under the heading *pestis major*, but there also occur mild forms to which the term *pestis minor* is applied. In these

latter there may be a moderate degree of swelling of a group of glands, attended with some pyrexia and general malaise, or there may be little more than slight discomfort. Between such and the graver types, cases of all degrees of severity are met with.

Experimental Inoculation.—Guinea-pigs, mice, rats, and rabbits are susceptible to inoculation, the first being on the whole most suitable for experimental purposes. After subcutaneous injection there occurs a local inflammatory œdema, which is followed by inflammatory swelling of the corresponding lymphatic glands, and thereafter by a general infection. The lesions in the lymphatic glands correspond in their main characters with those in the human subject, although usually at the time of death they have not reached a stage so advanced. By this method of inoculation mice usually die in one to three days, guinea-pigs and rats in two to five days, and rabbits in four to seven days. *Post mortem* the chief changes, in addition to the glandular enlargement, are congestion of internal organs, sometimes with hæmorrhages, and enlargement of the spleen; the bacilli are numerous in the lymphatic glands and usually in the spleen (Fig. 138), and also, though in somewhat less degree, throughout the blood. Infection can also be produced by smearing the material on the conjunctiva or mucous membrane of the nose, and this method of inoculation has been successfully applied in cases where the plague bacilli are present along with other virulent organisms, *e.g.* in sputum along with pneumococci. Rats and mice can also be infected by feeding either with pure cultures or with pieces of organs from cases of the disease, though in this case infection probably takes place through the mucous membrane of the mouth and adjacent parts, and only

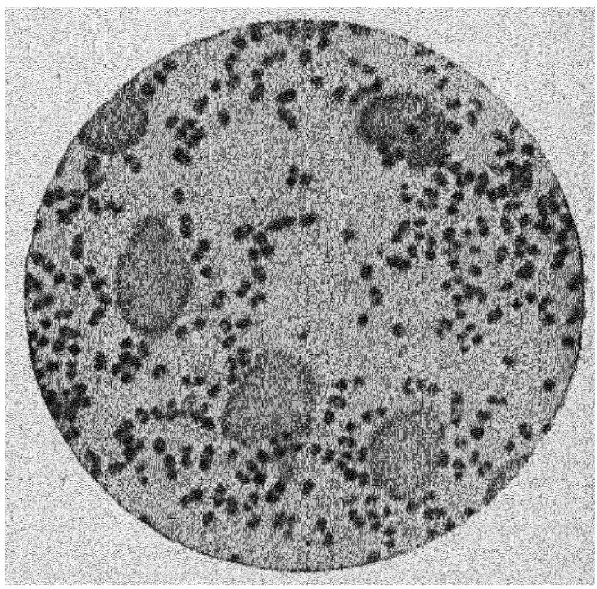


FIG. 138.—Film preparation of spleen of rat after inoculation with the bacillus of plague, showing numerous bacilli, most of which are somewhat plump. Stained with carbol-thionin-blue. ×1000.

to a limited extent, if at all, by the alimentary canal. Monkeys also are highly susceptible to infection, and it has been shown in the case of these animals that when inoculation is made on the skin surface, for example, by means of a spine charged with the bacillus, the glands in relation to the part may show the characteristic lesion and a fatal result may follow without there being any noticeable lesion at the primary seat. This fact throws important light on infection by the skin in the human subject.

Paths and Modes of Infection.—Plague bacilli may enter the system by the skin surface through small wounds, abrasions, etc., and in such cases there is usually little or no reaction at the site of entrance. This last fact is in accordance with what has been stated above with regard to experiments on monkeys. The path of infection is shown by the primary buboes, which are usually in the glands through which the skin is drained, those in the groin being the commonest site. Absolute proof of the possibility of infection by the skin is supplied by several cases in which the disease has been acquired at post-mortem examinations; in the majority of these the lesions of the skin surface were of trifling nature, and there was no local reaction at the site of inoculation. It may now, however, be regarded as established that the ordinary mode of skin infection is by means of the bites of fleas transmitting the bacilli from infected rodents, *e.g.* rats. It had previously been shown that when fleas were allowed to feed on animals suffering from plague, plague bacilli might be found for some time afterwards in the stomach, and some observers, for example Simond, had succeeded in transmitting the disease to other animals by means of the infected insects. Most observers, however, had obtained negative results, and it was only by the work of the Advisory Committee referred to above that the importance of this means of infection was established. By carefully planned experiments, the Committee showed that the disease could be transmitted from a plague rat to a healthy rat, kept in adjacent cages, when fleas were present; whereas this did not occur when means were taken to prevent the access of fleas, though the facilities for aerial infection were the same. The disease can also be produced by fleas removed from plague rats and transferred directly to healthy animals, success having been obtained in fully 50 per cent. of experiments of this kind. When plague-infected guinea-pigs are placed amongst healthy guinea-pigs, comparatively few of the latter acquire the disease when fleas are absent or scanty; whereas all of them may die of plague

when fleas are numerous. This result demonstrates the comparatively small part played by direct contact, even when of a close character. Important results were also obtained with regard to the mode of infection in houses where there had been cases of plague. It was found possible to produce the disease in susceptible animals by means of fleas taken from rats in plague houses. When animals were placed in plague houses and efficiently protected from fleas they remained healthy; whereas they acquired the disease when the cages were free to the access of fleas in the neighbourhood.

The following are some of the experiments which were conducted: A series of six huts were built which only differed in the structure of their roofs. In two the roofs were made of ordinary native tiles in which rats freely lodge; in two others, flat tiles were used in which rats live, but in which they have not such facilities for movement as in the first set, and in the third pair the roof was formed of corrugated iron. Under the roof in each case was placed a wire diaphragm which prevented rats or their droppings having access to the hut, but which would not prevent fleas falling down on to the floor of the hut. The huts were left a sufficient time to become infected with rats, and then on the floor in each case healthy guinea-pigs mixed with guinea-pigs artificially infected with plague were allowed to run about together. In the first two sets of huts to which fleas had access the healthy guinea-pigs contracted plague, while in the third set they remained unaffected, though they were freely liable to contamination by contact with the bodies and excreta of the diseased animals. In the third set of huts no infection took place as long as fleas were excluded, but when accidentally these insects obtained admission, then infection of the uninoculated animals commenced. Other experiments were also performed. In one case healthy guinea-pigs were suspended in a cage 2 inches above a floor on which infected and flea-infested animals were running about. Infection of the animals in the cage occurred; but if the cage were suspended at a distance above the floor higher than a flea could jump, then no infection took place. Again, in a hut in which guinea-pigs had died of plague, and which contained infected fleas, two cages were placed, each containing a monkey. One cage was surrounded by a zone of sticky material broader than the jump of a flea, another was left without this protection. The monkey in the former cage remained unaffected, but the other monkey contracted plague.

Other experiments showed that when plague bacilli were placed on the floors of houses, they died in a comparatively short period of time. After forty-eight hours it was not found possible to reproduce plague by inoculation with material from floors which had been grossly contaminated with cultures of the bacillus. Afterwards, however, animals placed in such a house might become infected by means of fleas. In all these experi-

ments the common rat-flea of India—*Xenopsylla cheopis*—was used, but it has been shown that this flea also infests and bites the human subject. Recent observations show that not only is plague transferable by means of fleas, but that this is practically the only method obtaining in natural conditions, with the exception that rats may become infected by eating the carcases of other animals containing large numbers of plague bacilli. It is improbable from the experiments made that bubonic plague is transmitted by direct contact even when of a close nature; in fact, it has been shown that plague-infected guinea-pigs may suckle their young without the latter acquiring the disease. *The general results show that in the bubonic type fleas in nearly all cases are the vectors of infection.*

The later work of the Committee supplied information of the highest value with regard to the epidemiology of the disease; it showed, in short, that plague in its epidemic form is dependent on the epizootic among rats, and with regard to this some further facts may be given. Plague in Bombay occurs in two chief species of rats, the *Rattus rattus*, the black house-rat, and *Rattus norvegicus* (*decumanus*), the common brown rat of the sewers. The former, owing to its presence in dwelling-houses, is chiefly responsible for the transmission of the disease to man; while the latter, on account of the large number of fleas which infest it, is of special importance in maintaining the disease from season to season. The year may be divided into two portions—an epizootic season, from December to May inclusive, and a non-epizootic, from June to November. During the latter period there are few cases of plague in rats on account of fleas being scanty; especially is this so in the case of *Rattus rattus*. In fact, in certain villages where this species alone is present, the disease may actually die out at the end of the epizootic season, and accordingly when plague reappears in these places this is due to a fresh importation—a fact of great practical importance. A fresh epizootic first affects chiefly *Rattus norvegicus*, and a little later spreads to *Rattus rattus*, while a little later still the disease attacks the human subject in the epidemic form; in each case fleas form the vehicle of transmission, and an interval of from ten to fourteen days intervenes between the outbreak of the epizootic and that of the epidemic. The proportion of cases of plague in *Rattus norvegicus* is much higher than in *Rattus rattus*, for the reason mentioned. It has been further shown that the bacilli flourish in the stomach of the flea and are passed in a virulent condition in the fæces, that a large proportion of the fleas removed from plague-infected

rats contain plague bacilli, and that the fleas may remain infective for a considerable number of days, sometimes for a fortnight. The subsidence of plague when the mean temperature rises above a certain level (about 80° F.) is probably in part, at least, due to the fact that the bacilli disappear much more rapidly from the alimentary tract of fleas at the higher temperatures; in accordance with this, experimental transmission of the disease to animals by means of fleas is more frequently successful at lower temperatures. Bacot and C. J. Martin have shown that infection occurs by regurgitation of infected blood from the stomach of the flea during the act of biting, the proventriculus being sometimes blocked by a mass of plague bacilli. The possibility of infection by contamination of the skin with the excrement of fleas containing the bacilli, however, cannot be excluded.

As regards the dying out of epidemics, some interesting facts have been brought forward by Liston. He and his co-workers have shown that rats taken from different towns vary greatly in their susceptibility to inoculation with plague bacilli, and that immunity is most marked in the rats from the towns which have suffered most severely from plague. This relative immunity appears to be due to the survival of the more resistant animals, and holds also with regard to their young. The diminution of plague amongst rats, and thus the subsidence of an epidemic, accordingly depends on the killing off of the more susceptible animals.

Enzootic and epizootic plague may occur in other wild rodent animals, *e.g.* the ground-squirrel in California, the gerbille and certain field-mice in South Africa, the tarabagan or Siberian marmot in Manchuria; and the infection in these animals may give rise to human cases or outbreaks.

In primary plague pneumonia, from a consideration of the anatomical changes and the clinical facts, the disease may be said to be produced by the direct passage of the bacilli into the respiratory passages by inhalation. The sputum droplets sprayed into the air by infected persons in the act of coughing carry the plague bacilli, which may survive for some time in cold wet weather, when the humidity of the atmosphere is high. In a dry atmosphere they die out quickly (Teague and Barber). Accordingly a case of plague pneumonia may be of great infectivity in producing other cases of plague pneumonia. Small epidemics of plague pneumonia break out from time to time, but in 1911 an extensive epidemic occurred in Manchuria leading to 50,000 deaths in six months. In this epidemic, direct infection

from patient to patient was clearly shown, and rats were not concerned in the spread. Plague pneumonia appears to occur first of all as a complication in a bubonic case, and there is no evidence that the bacilli differ in virulence in the two conditions.

Toxins, Immunity, etc.—As is the case with most organisms which extensively invade the tissues, the toxins in plague cultures are chiefly contained in the bodies of the bacteria. Injection of dead cultures in animals produces distinctly toxic effects; *post mortem*, hæmorrhage in the mucous membrane of the stomach, areas of necrosis in the liver, and at the site of inoculation, may be present. The toxic substances are comparatively resistant to heat, being unaffected by an exposure to 65° C. for an hour. By the injection of dead cultures in suitable doses, a certain degree of immunity against the living virulent bacilli is obtained, and, as first shown by Yersin, Calmette, and Borrel, the serum of such immunised animals confers a degree of protection on small animals such as mice. On these facts the principles of preventive inoculation and serum treatment, presently to be described, depend. It may also be mentioned that the filtrate of a plague culture possesses a very slight toxic action, and the Indian Plague Commission found that such a filtrate has practically no effect in the direction of conferring immunity.

1. *Preventive Inoculation—Haffkine's Method.*—To prepare the vaccine, cultures are made in flasks of bouillon with drops of oil on the surface (in India Haffkine employed a medium prepared by digesting goat's flesh with hydrochloric acid at 140° C. and afterwards neutralising with caustic soda). In such cultures stalactite growths (*vide supra*) form, and the flasks are shaken every few days so as to break up the stalactites and induce fresh crops. The flasks are kept at a temperature of about 25° C., and growth is allowed to proceed for about six weeks. At the end of this time sterilisation is effected by exposing the contents of the flasks to 65° C. for an hour; thereafter carbolic acid is added in the proportion of 0·5 per cent. The contents are well shaken to diffuse thoroughly the sediment in the fluid, and are then distributed in small sterilised bottles for use. The vaccine thus contains both the dead bodies of the bacilli and any toxins which may be in solution. It is administered by subcutaneous injection in the dose prescribed. Usually only one injection is made, sometimes two, though the latter procedure does not appear to have any advantage. The method has been systematically tested by inoculating a certain proportion of the inhabitants of districts exposed to infection, leaving others uninoculated, and then observing the proportion of cases of disease and the mortality

amongst the two classes. The results of inoculation have been distinctly satisfactory. For although absolute protection is not afforded by inoculation, both the proportion of cases of plague and the percentage mortality amongst these cases have been considerably smaller in the inoculated as compared with the uninoculated. Protection is not established till some days after inoculation, and lasts for a considerable number of weeks, possibly for several months (Bannerman). In the Punjab during the season 1902–03 the case incidence among the inoculated was 1·8 per cent., among the uninoculated 7·7 per cent., while the case mortality was 23·9 and 60·1 per cent. respectively in the two classes, the statistics being taken from villages where 10 per cent. of the population and upwards had been inoculated.

Plague vaccines have also been prepared from cultures on solid medium in accordance with the general methods used for vaccine preparation. There is no doubt that the use of living non-virulent cultures as vaccines produces a higher degree of immunity than results from injecting killed organisms. But the risk of setting up the disease in susceptible persons has prevented such living vaccines from being employed.

2. *Anti-plague Sera.*—Of these, two have been used as therapeutic agents, namely, that of Yersin and that of Lustig. Yersin's serum is prepared by injections of increasing doses of plague bacilli into a horse. In the early stages of immunisation dead bacilli are injected subcutaneously, thereafter into the veins, and, finally, living bacilli are injected intravenously. After a suitable time blood is drawn off and the serum is preserved in the usual way. Of this serum 10 to 20 c.c. are used, and injections are usually repeated on subsequent days. Lustig's serum is prepared by injecting a horse with repeated and increasing doses of a substance derived from the bodies of plague bacilli, probably in great part nucleo-protein. Masses of growth are obtained from the surface of agar cultures, and are broken up and dissolved in a 1 per cent. solution of caustic potash. The solution is then made slightly acid by hydrochloric acid, when a bulky precipitate forms; this is collected on a filter and dried. For use, a weighed amount is dissolved in a weak solution of carbonate of soda and then injected. The serum is obtained from the animal in the usual way. Extensive observations with both of these sera show that neither of them can be considered a powerful remedy in cases of plague, though in certain instances distinctly favourable results have been recorded. The Indian Commission, however, came to the conclusion "that, on the whole, a certain amount of advantage accrued to the patients in cases both of those injected with Yersin's serum and of those injected with Lustig's serum" It may also be mentioned that the Commission found, as the result of experiments, that Yersin's serum modified favourably the course of the disease in animals, whereas Lustig's serum had no such effect.

Serum Diagnosis.—Specific agglutinins may appear in the blood

of patients suffering from plague, as also they do in the case of animals immunised against the plague bacillus. It is to be noted, however, that in clinical cases the reaction is not invariably present, the potency of the serum is not of high order, and the carrying out of the test is complicated by the natural tendency of the bacilli to cohere in clumps. For the last reason the macroscopic (sedimentation) method is to be preferred to the microscopic (p. 125). A suspension of plague bacilli is made by breaking up a young agar culture in 0·75 per cent. sodium chloride solution; the larger flocculi of growth are allowed to settle, and the fine, supernatant emulsion is employed in the usual way. According to the results of the German Plague Commission and the observations of Cairns, made during the Glasgow epidemic, it may be said that the reaction is best obtained with dilutions of the serum of from 1 : 10 to 1 : 50. Cairns found that the date of its appearance is about a week after the onset of illness, and that it usually increases till about the end of the sixth week, thereafter fading off. It is most marked in severe cases characterised by an early and favourable crisis, less marked in severe cases ultimately proving fatal, whilst in very mild cases it is feeble or may be absent. The method, if carefully applied, may be of service under certain conditions; but it will be seen that its use as a means of diagnosis is restricted. The use of high-titre agglutinating antisera obtained from horses is of great value for identifying cultures of B. pestis.

Methods of Diagnosis.—Where a bubo is present a little of the "juice" may be obtained by puncture and aspiration with a sterile hypodermic syringe. The fluid is then to be examined microscopically, and cultures on agar or serum agar should be made by the successive stroke method. The cultural and morphological characters are then to be investigated, the most important being the involution forms on salt agar and the stalactite growth in bouillon, though the latter may not always be obtained with the plague bacillus. The pathogenic properties should also be studied, the guinea-pig being on the whole most suitable for subcutaneous inoculation. In many cases a diagnosis may be made by microscopic examination alone, as in no known condition other than plague do bacilli with the morphological characters of the plague bacillus occur in large numbers in the lymphatic glands. The organism may be obtained in culture from the blood in a considerable proportion of cases by withdrawing a few cubic centimetres and proceeding in the usual manner. On the occurrence of the first suspected case, every care to exclude possibility of doubt should be used before a positive opinion is given.

In a case of suspected plague pneumonia, in addition to microscopic examination of the sputum, the above cultural methods along with animal inoculation with the sputum should be carried out; smearing the nasal mucous membrane or a freshly shaved area of skin of a guinea-pig may be recommended. Here a positive diagnosis should not be attempted by microscopic examination alone, especially in a plague-free district, as bacilli morphologically resembling the plague organism may occur in the sputum in other conditions.

Recognition of Plague Infection in Rats and other Wild Rodents.—This is of great importance in plague prevention. In the autopsy of the suspected animal careful attention is first paid to the

various groups of lymph glands, particularly those of the neck which are most frequently affected. The glands show marked enlargement with surrounding œdema and often hæmorrhages, and plague bacilli may be demonstrated in smears from the tissue. The spleen is enlarged and the liver shows often a characteristic mottling due to yellow necrotic areas. There is usually marked congestion of the subcutaneous vessels, often with hæmorrhages. Effusion into the pleuræ may be noted. In an animal recently dead after an acute infection the plague bacilli may be demonstrated in the heart blood and spleen. It often happens, however, that carcases of rats found dead and requiring examination for plague infection are decomposed, and it may be impossible to demonstrate the bacilli microscopically and isolate them by the usual methods of culture. The existence of the infection may often be established in such cases by inoculating a shaved and scarified area of skin in a guinea-pig with material from the lesions. The plague bacilli invade the tissues and produce a typical plague infection.

Pasteurella Group. — It is noteworthy in reference to the bacteriology of plague, that the B. pestis is a member of a large group of bacteria (designated the Pasteurella group) which are responsible for epizootic disease among various animal species. Organisms of this group are causally associated with the condition of hæmorrhagic septicæmia among fowls (B. avisepticus), rabbits (B. cuniculisepticus) (Fig. 139), cattle (B. bovisepticus), pigs (B. suisepticus), etc. These organisms all present morphological and cultural characters similar to that of the plague bacillus. In the investigation of plague among animals, the differentiation of B. pestis from other organisms of the group may be of considerable importance. It was shown by the Indian Plague Commission that the hæmorrhagic septicæmia organisms are incapable of growing in media containing sodium taurocholate (*e.g.* MacConkey's medium), while the B. pestis can be grown on this medium. An investigation of the pathogenicity and effects of strains in question under experimental conditions in different laboratory animals should also be carried out, as well as the agglutination reaction with a potent antiserum.

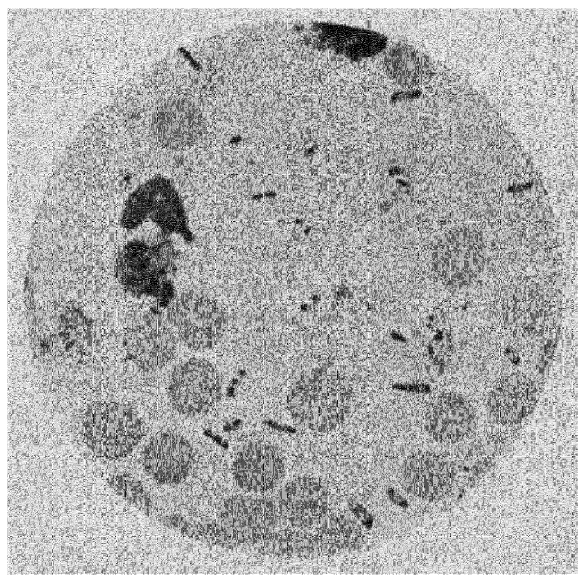


FIG. 139.—Film preparation from lung of rabbit, dead of pasteurella infection. ×1000. (Leishman's stain.)

TULARÆMIA

Bacterium Tularense (*Pasteurella tularensis*).—This organism was first described by M'Coy and Chapin in a plague-like disease

of ground-squirrels in California. The infection also occurs in other wild rodents, *e.g.* rabbits. The organism is a very minute bacilloid or coccoid body measuring 0·3 μ to 0·7 μ in its longest diameter. It stains Gram-negatively and in films from animal lesions often presents the appearance of a capsule. It can be demonstrated microscopically in the spleen of infected animals where it may be present in enormous numbers. Artificial cultures were first obtained on coagulated egg-yolk. More recently the organism has been cultivated on blood-agar and serum-glucose-agar containing a piece of sterile rabbit's spleen.

The infection, spoken of as tularæmia, is transmissible to man, and a considerable number of cases in America have now been recorded. Certain of these have resulted from contact with infected animals, *e.g.* through handling diseased rabbits and other rodents. It is likely also that cases may arise from the bite of a blood-sucking insect. It is specially remarkable how readily laboratory workers may become infected by handling infective material and artificial cultures, and cases of such laboratory infection have occurred both in America and in this country, leading to a somewhat protracted period of invalidism. Artificial cultures apparently retain a remarkably high degree of virulence and infectivity. Laboratory animals can be infected experimentally from cultures, and at autopsy the principal pathological changes are multiple necrotic foci in the spleen, liver, and lymph nodes. The pathological picture varies considerably in different organs and in different species. According to Ledingham, the spleens of infected guinea-pigs dead of tularæmia may show characteristic lesions along with diffuse polymorpho-nuclear leucocyte infiltration. In such specimens the organisms cannot be demonstrated microscopically. On the other hand, in mice the spleen may show a much lesser degree of pathological change with no leucocytic response and films from the tissue contain large numbers of organisms. Ledingham has also noted that in mice the cells of the liver parenchyma are invaded by the organism in large numbers, and the cell cytoplasm is often replaced by them. It has been found that the infection may be passed experimentally to laboratory animals by infected blood-sucking insects.

In human cases of laboratory infection, it has been found that the serum agglutinates the bacterium tularense in dilutions up to 1 : 800 (Ledingham).

CHAPTER XX

MALTA FEVER AND EPIZOOTIC ABORTION

Malta or Undulant Fever

This disease is of common occurrence along the shores of the Mediterranean and in its islands. Since its bacteriology has been worked out, it has been found to occur also in India, China, South Africa, and in some parts of North and South America, its distribution being much wider than was formerly supposed. Although from its symptomatology and pathological anatomy it had been recognised as a distinct affection, and was known under various names, its precise etiology was unknown till the publication of the researches of Bruce in 1887. From the spleen of patients dead of the disease he cultivated a characteristic organism, now known as the *Bacillus melitensis*, and by means of inoculation experiments established its causal relationship to the disease. Wright and Semple applied the agglutination test to the diagnosis of the disease, and in 1904 the mode of spread of the disease was fully studied by a Commission, whose work demonstrated that goat's milk is the chief source of infection.

The duration of the disease is usually long—often two or three months, though shorter and much longer periods are met with. Its course is very variable, the fever being of the continued type with irregular remissions. In addition to the usual symptoms of pyrexia, there occur profuse perspiration, pains and sometimes swellings in the joints, occasionally orchitis, whilst constipation is usually a marked feature. The mortality is low—about 2 per cent. (Bruce).

In fatal cases the most striking post-mortem change is in the spleen. This organ is enlarged, often weighing slightly over a pound, and in a condition of acute congestion ; the pulp is soft and may be diffluent, and the Malpighian bodies are swollen and indistinct. In the other organs the chief change is cloudy swelling ; in the kidneys there may be in addition glomerular nephritis. The lymphoid tissue of the intestines shows none of the changes characteristic of typhoid fever.

Bacillus Melitensis (*Alcaligenes melitensis*).—This is a small, rounded, oval or cocco-bacillary organism about 0·4 μ in diameter, which is specially abundant in the spleen. It usually occurs singly or in pairs, but in cultures short chains are also met with (Fig. 140). The coccal form of the organism originally led to its being designated a micrococcus. In culture, however, its bacillary character is more obvious. It stains fairly readily with the ordinary basic aniline stains, but loses the stain in Gram's method. It is non-motile. In the spleen of a patient dead of the disease it occurs irregularly scattered through the congested pulp; it may also be found in small numbers *post mortem* in the capillaries of various organs. It may be cultivated from the blood during life in a considerable proportion of cases. The organism was found by the members of the Commission in the urine of Malta fever patients in 10 per cent. of the cases examined; it was sometimes scanty, but sometimes present in large numbers. It has also occasionally been obtained from the fæces.

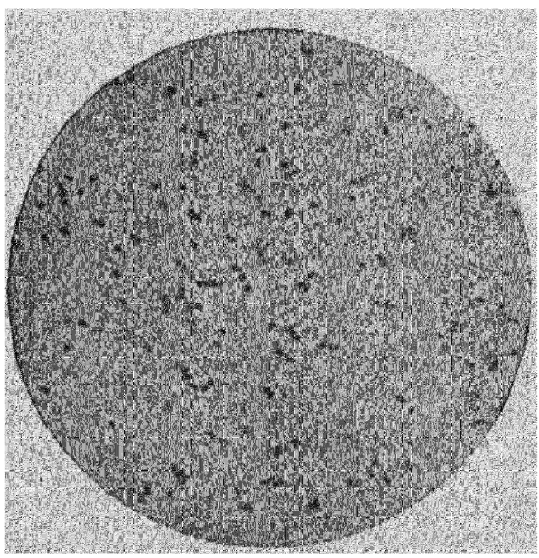


FIG. 140.—Bacillus melitensis, from a two days' culture on agar at 37° C. Stained with fuchsin. ×1000.

Cultivation.—Growth occurs on ordinary media, *e.g.* agar, at 37°C. In primary culture, the colonies, which are usually not visible before the third or fourth day, appear as small round discs, slightly raised and of somewhat transparent appearance. The maximum size—2 to 3 mm. in diameter—is reached about the ninth day; at this period by reflected light they appear pearly white, while by transmitted light they have a yellowish tint in the centre, bluish-white at the periphery. After being accustomed to artificial medium the organism grows more abundantly and more rapidly. A stroke culture shows a layer of growth of similar appearance with somewhat serrated margins. The optimum temperature is 37° C., but growth still occurs down to about 20° C. In gelatin at summer temperature growth is extremely slow—after two or three weeks, in a puncture culture, there is a delicate line of growth along the

needle track and a small flat expansion of growth on the surface. There is no liquefaction of the medium. In bouillon there occurs a general turbidity with flocculent deposit at the bottom; on the surface there is no formation of a pellicle. The reaction of the media ought to be very faintly alkaline, as marked alkalinity interferes with the growth; a reaction of +10 (p. 43) has been found very suitable. On potato no visible growth takes place even at the body temperature, though the organism multiplies to a certain extent. Growth on various sugar media fails to reveal any fermentative properties. Outside the body the organism has considerable powers of vitality, as it has been found to survive in a dry condition in dust and clothing for a period of two months.

Relations to the Disease.—There is in the first place ample evidence, from examination of the spleen, both *post mortem* and during life, that this organism is always present in the disease. The experiments of Bruce and Hughes first showed that by inoculation with even comparatively small doses of pure cultures the disease could be produced in monkeys, sometimes with a fatal result. And it has now been fully established that inoculation with the minutest amount of culture, even by scarification, leads to infection both in monkeys and in the human subject.

Rabbits, guinea-pigs, and mice are, as a rule, insusceptible to inoculation by the ordinary method, though some strains may produce pathogenic effects. Durham, by using the intracerebral method of inoculation, however, succeeded in raising the virulence, so that the organism was capable of producing in guinea-pigs on intraperitoneal injection illness with sometimes a fatal result many weeks afterwards. Eyre also, by increasing the virulence by intracerebral inoculation, was able to produce infection in various animals, especially on intravenous injection.

Mode of Spread of the Disease.—The work of the 1904 Commission resulted in establishing facts of the highest importance with regard to the spread of the disease. In the course of investigations Zammitt found that the blood serum of many of the goats agglutinated the bacillus melitensis, and Horrocks obtained cultures of the organism from the milk. Further observations showed that the agglutination reaction occurred in the case of 50 per cent. of the goats in Malta, whilst the organism was present in the milk in 10 per cent. Sometimes it was present in enormous numbers, and in these cases the animal usually appeared poorly nourished, whilst the milk had a somewhat serous character. In other cases, however, it was present

when the animals appeared healthy, and there was no physical or chemical change discoverable in the milk. It was also determined that the organism might be excreted for a period of two to three months before any notable change occurred in the milk. The milk of infected animals usually agglutinated the bacillus, and this property was always present when the bacillus was found in the milk. It was, moreover, found that monkeys and goats could be readily infected by feeding them with milk containing the organism, the disease being contracted by fully 80 per cent. of the monkeys used. It was therefore rendered practically certain that the human subject was infected by means of such milk, and the result of preventive measures, by which milk was excluded as an article of dietary amongst the troops in Malta, fully bore out this view. After such measures were instituted, the number of cases in the second half of 1906 fell to 11 per thousand, as contrasted with 47 per thousand in the corresponding part of the preceding year; cases now are relatively few. Various facts with regard to the epidemiology of the disease were thus cleared up. For example, it was more prevalent in the summer months, when more milk was consumed; and there was a larger proportion of cases amongst those in good social position, the officers, for example, suffering more in proportion than the rank and file. Another interesting fact, pointed out by Horrocks, is that the disease practically disappeared from Gibraltar after the practice of importing goats from Malta was stopped. The manner in which the disease spreads from goat to goat has not yet been satisfactorily determined.

The work of the Commission excluded other modes of infection than the ingestion of infected milk as being of practical importance; if the disease is conveyed by contact at all, this is only when the contact is of an intimate character, and even then it is probably of rare occurrence.

There is distinct evidence that the disease may be acquired by inoculation through small lesions in the skin, and this method is probably not infrequent amongst those who handle infected milk. It has been shown that the organism may remain alive in the bodies of mosquitoes for four or five days, and it might seem theoretically possible that insects may occasionally be the means of carrying the disease; there is no evidence, however, that this takes place to any extent.

Reference is made later, under B. abortus, to the question of the relationship of this organism to undulant fever in the human subject.

Agglutination Reaction.—The blood serum of patients suffering from Malta fever possesses the power of agglutinating the bacillus melitensis in a manner analogous to what has been described in the case of typhoid fever ; here also dead cultures may be used. The reaction appears comparatively early, often about the fifth day, and may be present for a considerable time after recovery—sometimes for more than a year. Normal serum may agglutinate B. melitensis occasionally in relatively high dilution, *e.g.* 1 : 100 and even 1 : 200 by the macroscopic method (p. 125). In the disease, however, the agglutination reaction usually occurs with much higher dilution, *e.g.* 1 : 500, or over. The normal serum effect is said to be destroyed at 55° C., whereas the specific agglutinin is not affected. Some observers accordingly recommend that, in applying the test, the serum ought to be first heated to 55° C. As regards relation to prognosis, the observations of Birt and Lamb and of Bassett-Smith have given results analogous to those obtained in typhoid (p. 420).

The Commission found that vaccination with dead cultures of the organism confers a certain degree of protection amongst those exposed to the disease. As a rule, two injections were given, 200–300 million organisms being the dose of the first injection, and about 400 million the dose of the second. The use of vaccines has also been carried out in the treatment of the disease, but the results are not sufficiently conclusive to establish its value.

Methods of Diagnosis.—During life the readiest means of diagnosis is supplied by the agglutination test just described (for technique, *vide* p. 124).

Cultures are most easily obtained from the spleen either during life by spleen puncture or *post mortem*. Inoculate a number of agar tubes by successive strokes and incubate at 37° C. Film preparations should also be made from the spleen pulp and stained with carbol-thionin-blue and Gram's method. Cultures may sometimes be obtained from the blood by the usual methods. In the identification of the organism agglutination by an anti-serum to a known strain would constitute conclusive proof, as in the methods used for the typhoid-paratyphoid group (*vide* p. 441). It is to be noted that in a small proportion of cases of Malta fever the causal organism has been stated to be a strain serologically different from the common type of B. melitensis and the name *B. paramelitensis* has been given to it.

Great care must be exercised in working with cultures of the B. melitensis, as bacteriologists have become infected apparently from such sources, in an unusually high proportion of instances.

Bacillus Abortus

Brief reference may be made here to the Bacillus abortus (*Alcaligenes abortus*) in view of its close biological similarity to the B. melitensis, its occurrence in cow's milk, and the possibility of its pathogenicity to the human subject. The organism was first described by Bang in 1897 as the cause of bovine infectious abortion. In morphology it is a very small, pleomorphic, non-motile, Gram-negative bacillus not exceeding 1 to 2 μ in length and 0·3 to 0·8 μ in breadth, and often coccal in form like B. melitensis. From material in which the organism is likely to be present in a state of purity, *e.g.* the heart blood of the fœtus, it may be cultivated, according to Bang's original method, in the form of a shake-culture in a tube of serum agar. The organism in primary growth is markedly micro-aerophilic (*vide* p. 20), and the colonies develop after inoculation in a zone about 10–20 millimetres below the surface of the medium. After continued cultivation the organism can be grown under aerobic conditions on ordinary agar, the colonies being small transparent discs resembling those of B. melitensis. When primary cultures are required from material likely to contain other organisms, *e.g.* from the genital passages of infected cows, cultivation presents greater difficulty and requires special methods which need not be detailed here, *e.g.* cultivation in a closed jar containing growths of B. subtilis to reduce the oxygen concentration (Evans). Smith has shown that it will grow readily, in first culture, in an atmosphere containing a carbon-dioxide concentration up to 10 per cent. Like B. melitensis, it has no fermentative properties on carbohydrates. It grows well in milk.

It is now well established that the B. abortus is responsible for bovine abortion, and its relationship to the condition has been proved by experimental infection in pregnant cows, *e.g.* by injection of cultures into the vagina Intravenous injection in pregnant rabbits and guinea-pigs may also lead to abortion in these animals. It is of particular interest and importance that experimental infection in guinea-pigs may produce tubercle-like lesions (Smith and Fabyan). The infection in cattle is usually associated with little general effects. The development of the fœtus is arrested, and there may be inflammation of the fœtal membranes. The organism may persist in the genital organs for some time after the abortion. The agglutination reaction of the serum with known cultures has been applied in the recognition of infected animals.

According to Evans, the B. abortus and B. melitensis are so closely related biologically that they can only be differentiated by agglutination reactions. It is also of interest that abortion has resulted in pregnant guinea-pigs inoculated experimentally with B. melitensis of human origin. There is a difference, however, in the virulence of B. melitensis and B. abortus to monkeys, the latter being less virulent. Evans has now studied a number of strains of the two types and has found that the majority of those of bovine and porcine origin, are serologically of the B. abortus type, but that a few strains of human origin also correspond to this organism. One strain of B. melitensis type was also isolated from an aborted bovine fœtus and another from a goat after abortion. Experimental abortion has also been produced in a cow by inoculation with a

strain of the melitensis type of human origin. These facts all tend to show the close relationship of the two organisms. Cases of undulant fever have been recorded in which it has been impossible to trace the infection to goat's milk, and the question has been raised as to the possibility of infection from a bovine source in view of the relationship of B. abortus to B. melitensis and the occurrence of the former in cow's milk. Keefer has recorded a case of undulant fever in Baltimore, due apparently to the B. abortus type, and his observations have been confirmed by Orpen in South Africa and by Evans in a case in Virginia. It is also of interest in this connection that in France, cases of undulant fever which could not be traced to goat's milk occurred in abattoir workers (Césari). These observations all go to show that the organism of bovine infectious abortion may, under certain circumstances, produce an infection in man of the nature of undulant fever, and the biological relationship of B. melitensis of human origin and B. abortus from a bovine source is paralleled, to some extent, in their pathogenicity for the human subject, though it seems likely that the B. abortus type is of lower virulence to man. The question also arises as to whether bovine infectious abortion is to be regarded as a condition of importance from the point of view of public health. Wilson and Nutt have recently shown that B. abortus may occur in a certain proportion of specimens of cow's milk; on the other hand, in spite of this, there is no evidence that this organism is responsible for human infections in this country.

Other organisms biologically allied to B. melitensis are the *Bacillus of guinea-pig pneumonia*, described by Smith, and the *B. bronchisepticus* reported by Ferry and by M'Gowan as associated with canine distemper.

CHAPTER XXI

TETANUS: CONDITIONS CAUSED BY OTHER ANAEROBIC BACILLI

Introductory.—Tetanus is a disease which in natural conditions affects chiefly man and the horse. Clinically it is characterised by the gradual onset of general stiffness and spasms of the voluntary muscles, commencing in those of the jaw and the back of the neck, and extending to all the muscles of the body. These spasms are of a tonic nature, and, as the disease advances, succeed each other with only a slight intermission of time. There are often, towards the end of a case, fever and rise of respiration and pulse-rate. The disease is usually associated with a wound received ordinarily from four to fourteen days previously, which has been defiled by earth or dung. The disease is, in the majority of cases, fatal.

Historical.—The general association of the development of tetanus with the presence of wounds, though these might be very small, suggested that some infection took place through the latter, but for long nothing was known as to the nature of this infection. Carle and Rattone in 1884 announced that they had produced the disease in a number of animals by inoculation with material from a wound in tetanus. They thus demonstrated the transmissibility of the disease. Nicolaier (1885) infected mice and rabbits with garden earth, and found that many of them developed tetanus. Suppuration occurred in the neighbourhood of the point of inoculation, and in this pus, besides other organisms, there was always present, when tetanus had occurred, a bacillus having certain constant microscopic characters. Inoculation of fresh animals with such pus reproduced the disease. Nicolaier's attempts at its isolation by the ordinary gelatin plate-culture method were, however, unsuccessful. He succeeded in getting it to grow in liquid blood serum, but always in mixture with other organisms. Infection of animals with such a culture produced the disease. These results were confirmed by Rosenbach, who, though failing to obtain a pure culture, cultivated the other organisms present, and inoculated them, but with negative results. He further pointed out, as characteristic of the bacillus, its development of terminal spores. In 1889, Kitasato succeeded in isolating from the local suppuration of mice inoculated from a human case, several bacilli, only one of which, when injected in pure culture into animals,

caused the disease, and which was now named the B. tetani. Kitasato found that the cause of earlier culture failures was the fact that it could only grow in the absence of oxygen. The pathology of the disease was further elucidated by Faber, who, having isolated bacterium-free poisons from cultures, reproduced the symptoms of the disease.

Bacillus Tetani (*Clostridium tetani*).—As seen in young cultures, the B. tetani appears as a slender organism, usually about 4 μ to 5 μ in length and about 0·4 μ in thickness, with somewhat rounded ends. Besides occurring as shorter rods, it also develops filamentous forms, the latter being more common in fluid media. It stains readily and uniformly by any of the usual stains and is Gram-positive. It is very slightly motile, and its motility can be best studied in an anaerobic hanging-drop preparation. When stained by the special methods already described, it is found to possess numerous delicate flagella attached both at the sides and at the ends (Fig. 143). These flagella, though they may be of considerable length, are usually curled up close to the body of the bacillus. The formation of flagella can be best studied in preparations made from surface anaerobic cultures (p. 79). As is the case with many other anaerobic flagellated bacteria, the flagella, on becoming detached, often become massed together in the form of spirals of striking appearance (Fig. 144). At incubation temperature B. tetani readily forms spores, and then presents a very characteristic appearance. The spores are round, and in diameter may be three or four times the thickness of the bacilli. They are developed at one end of a bacillus, which thus assumes what is usually described as the "drumstick" form (Figs. 141, 142). In a specimen stained with a watery solution of methy-

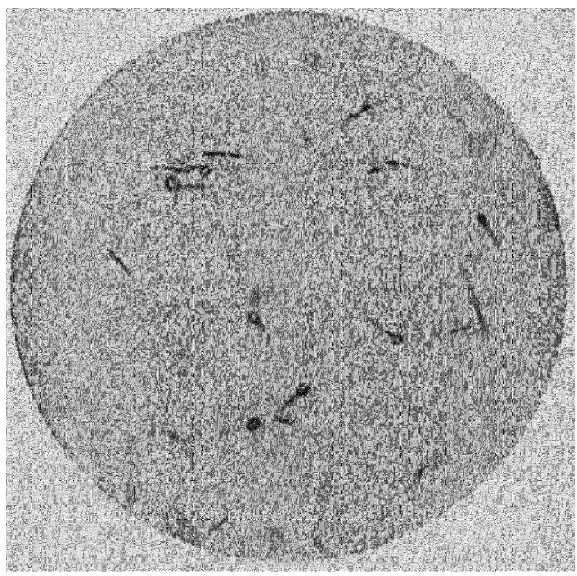


FIG. 141.—Tetanus bacilli; some of which possess spores. From a culture in glucose agar, incubated for three days at 37° C. See also Plate IV., Fig. 20. Stained with carbol-fuchsin. ×1000.

lene-blue, the spores are uncoloured except at the periphery, so that the appearance of a small ring is produced; if a powerful stain such as carbol-fuchsin be applied for some time, the spores become deeply coloured like the bacilli. In the discharge from a wound infected with B. tetani, both spore-bearing and spore-

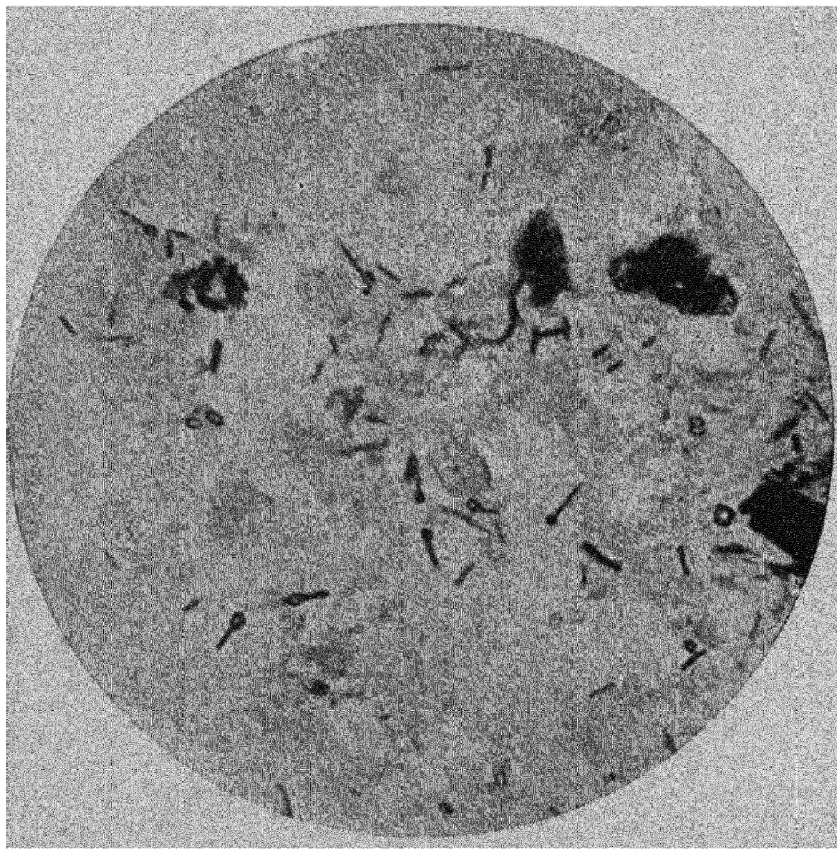


FIG. 142.—Film preparation of discharge from wound in a case of tetanus, showing several tetanus bacilli of "drumstick" form. (The thicker bacillus present is not a tetanus bacillus, but a putrefactive anaerobe which was obtained in pure culture from the wound.)

Stained with gentian-violet. ×1000.

free forms are usually to be found, the latter having no distinctive features.

Occurrence in Nature. — By inoculation experiments in animals, the natural habitat of B. tetani has been proved to be richly manured soil, and especially the contents of dung-heaps, where it probably leads a saprophytic existence, though its function as a saprophyte is unknown. It also occurs in the dust of houses, on the skin and in the intestines of many—especially of herbivorous—animals, and has been recovered

from the intestine of man. From such sources and from the pus of wounds in tetanus, occurring naturally or experimentally produced, it has been isolated by means of the methods appropriate for anaerobic bacteria.

Isolation.—(1) The principle is to take advantage of the resistance of the spores of the bacillus to heat. A sloped tube of inspissated serum or a deep tube of glucose agar is inoculated and incubated anaerobically at 37° C. for forty-eight hours, at the end

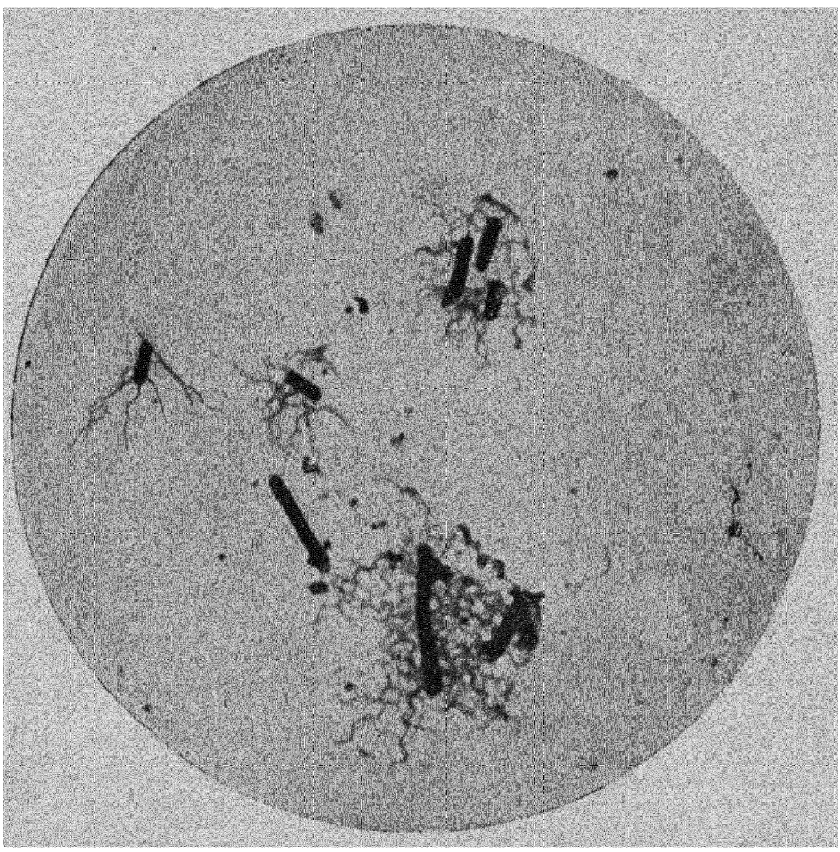


FIG. 143.—Tetanus bacilli, showing flagella. Stained by Rd. Muir's method. ×1000.

of which time numerous spore-bearing bacilli can often be observed microscopically. The culture is then kept at 80° C. for from three-quarters to one hour, with the view of killing all organisms except those which have spored. From such material agar anaerobic plates are prepared by one of the methods described on p. 80 *et seq.* This method of isolation is frequently unsuccessful, because along with the tetanus bacilli, both in its natural habitats outside the body and in the pus of wounds, other spore-forming obligatory and facultative anaerobes occur, which grow faster than the tetanus bacillus, and thus overgrow it.

(2) The method by which Fildes has isolated many strains depends upon the fact that under suitable conditions B. tetani

grows as a spreading film which extends beyond the growth of other organisms. With material from a case of tetanus it is advisable to enrich the bacilli first by inoculating a tube of freshly boiled blood broth [1] which is incubated aerobically at 37° C. for two to four days. Then from this culture the condensation water of a sloped tube of digest blood agar is inoculated and incubated anaerobically at 37° C. After twenty-four to forty-eight hours the extreme edge of the film of growth seen with a hand-lens as a tangle of extremely fine filaments, is found on microscopic examination to consist of a culture of the tetanus bacillus; subcultures from the margin yield pure growths of the organism. The detection of the edge of the growth is facilitated by using agar tubes which have

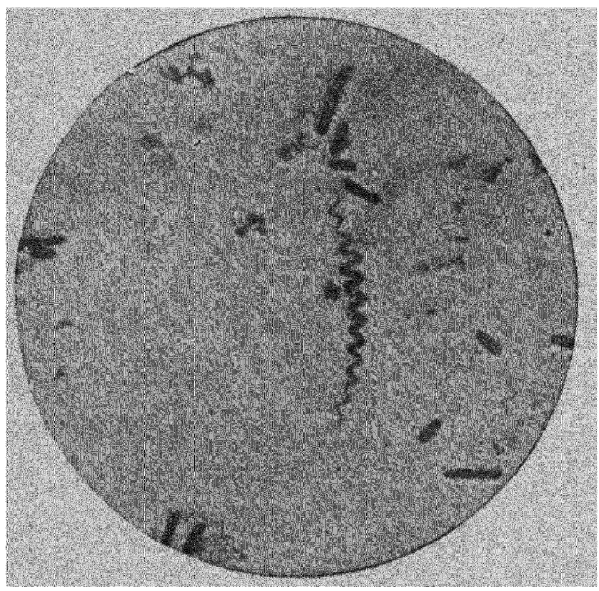


FIG. 144.—Spiral composed of numerous twisted flagella of the tetanus bacillus.
Stained by Rd. Muir's method. ×1000.

been kept until the medium has become slightly dry at the top. The only organism which is likely to spread as extensively as B. tetani is Bacillus proteus. The latter can be eliminated by heating the mixed growth containing spores of B. tetani.

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[1] Peptic blood digest is prepared by keeping a mixture of 150 c.c. saline, 6 c.c. pure HCl, 50 c.c. defibrinated sheep's blood, and 1 gram pepsin, B.P. granulated, in a well-stoppered bottle at 55° C. in the water bath for two to twenty-four hours. Then add 20 per cent. NaOH solution (about 12 c.c. are required) till a sample of the mixture diluted with water gives a permanganate-red colour with cresol-red indicator. Now add pure HCl drop by drop till a sample gives almost no change of colour with cresol-red, but gives a red tint with phenol-red (avoid excess of acid). 0·25 per cent. of chloroform is added to the mixture and dissolved by shaking. This digest keeps for many months; before use the mixture should be shaken and 2 to 5 per cent. added with a sterile pipette to broth or agar (Fildes, *Brit. Journ. Exper. Path.*, 1925, vi. 62).

Characters of Cultures and Conditions of Growth.—In the case of the ordinary media growth takes place only in the absence of free oxygen, the organism being an *anaerobe*; but when particulate matter is present, *e.g.* in minced meat medium, B. tetani like other anaerobes will grow without exclusion of the air. In deep *glucose gelatin* (in which growth is often very difficult to obtain) there commences, an inch or so below the surface, a growth consisting of fine straight threads, rather longer in the lower than in the upper parts of the tube, radiating out from the needle track (Fig. 145). Slow liquefaction of the gelatin takes place, with slight gas formation. In *agar* the growth is somewhat similar, consisting of small nodules along the needle track, with irregular short offshoots passing out into the medium (Fig. 153, A). There is slight formation of gas, but, of course, no liquefaction. On anaerobic agar plates colonies have under a low power a feathery outline (Fig. 146). Kitasato compared the colonies in gelatin plates to those of the B. subtilis. They consist of a thick centre with shoots radiating out on all sides. They liquefy the gelatin more slowly than the B. subtilis. Growth occurs in *blood serum* and *glucose bouillon* under anaerobic conditions and also in Robertson's *bullock's heart medium*. There is in it at first a slight turbidity, and later a thin layer of a powdery deposit on the walls of the vessel. All the cultures give out a peculiar burnt odour of rather unpleasant character. Meat medium undergoes no change in colour. None of the sugars commonly used are fermented. Serum is only slightly liquefied. In making subcultures on fluid media a considerable amount of the original growth should be used for the inoculation.

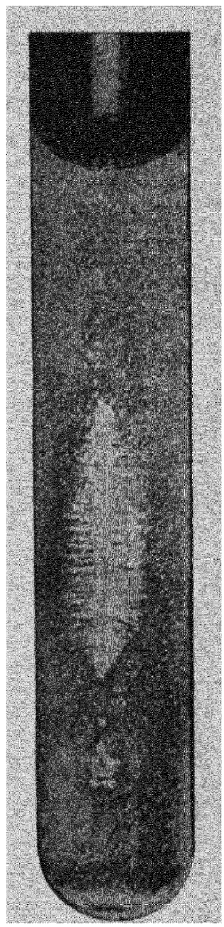


FIG. 145.—Stab culture of the tetanus bacillus in glucose gelatin, showing the lateral offshoots (after Kitasato). Natural size.

The B. tetani grows best at 37° C. The minimum growth temperature is about 14° C., and below 22° C. growth takes place very slowly. Sporulation may commence at the end of twenty-four hours in cultures grown at 37° C.—much later at

lower temperatures. Like other spores, those of tetanus are extremely resistant. They can usually withstand boiling for five minutes, and can be kept in a dry condition for many months

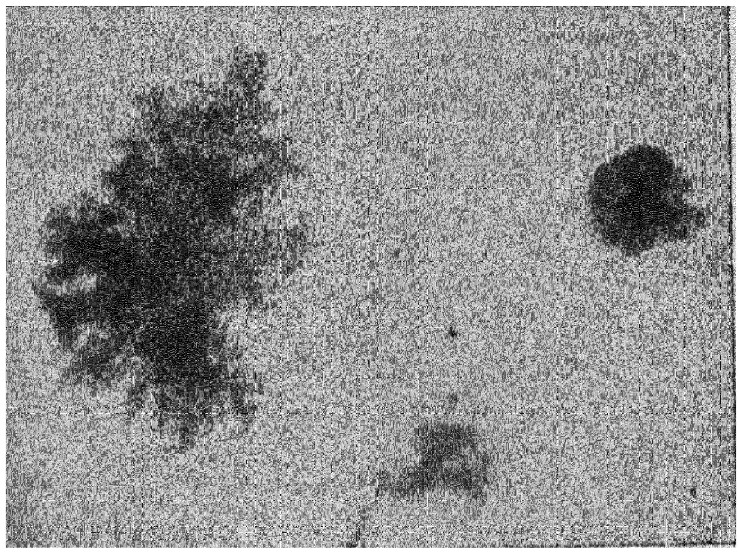


FIG. 146.—Colonies of the tetanus bacillus on anaerobic agar plates, seven days old. ×50.

without being killed or losing their virulence. They have also high powers of resistance to antiseptics.

Pathogenic Effects.—The proof that the B. tetani is the cause of tetanus is complete. It can be isolated in pure culture, and when re-injected in pure culture it reproduces the disease. It may be impossible to isolate it from some cases of the disease, but the cause of this very probably is the small numbers in which it sometimes occurs. The tetanus bacillus lacks the power of invading the tissues by itself. It obtains a foothold only under favouring conditions such as are afforded by a mixed infection with pyogenic organisms, the presence of foreign matter or necrotic tissue. That this is the case appears from experiments of Vaillard and others in which tetanus spores freed from toxin by heating at 80° C. were injected. The animals remained healthy, but the spores persisted in a living state for a considerable time, and tetanus was set up in guinea-pigs when, after some weeks, staphylococci were injected subcutaneously into the same site as the tetanus spores. Similarly in rabbits after intravenous injection of such detoxicated spores a simple fracture of the femur caused the disease to develop. Tetanus bacilli tend to remain localised at the site of entry in

a wound, and the toxin which they produce is absorbed and acts on the central nervous system.

(*a*) *The Disease as arising naturally.*—It occurs chiefly in horses and in man. Other animals may, however, be affected. In different animal species variations in the clinical progress of the disease are observed. In man and in the horse the spasms early affect the extensor muscles of the trunk (descending tetanus), while in small animals they usually appear first in the muscles neighbouring on the site of infection (ascending tetanus). There is in most cases a definite wound, often of a ragged character, which has either been made by an object soiled with earth or dung, or which has become contaminated with these substances. There is often a purulent or fœtid discharge, though this may be absent. In tetanus following clean operation wounds, catgut ligatures may be the source of infection. It is important to note that the wound through which infection has taken place may be very small, in fact, may consist of a mere abrasion. In some cases, especially in the tropics, it may possibly be merely the bite of an insect. In some parts of the world infection through the umbilicus originates a high mortality from the disease in newly born infants. The absence in many cases of an obvious channel of infection has given rise to the term " idiopathic " tetanus.

During the recent war the clinical type of tetanus seen in the wounded has been modified in consequence of the wide application of the prophylactic injection of anti-tetanus serum (*vide infra*). In the first place there has been a tendency to the prolongation of the incubation period, instances where this has extended to many months being not uncommon. In such cases there has usually been an unhealed septic wound, often containing foreign bodies, and the attack of tetanus may be precipitated by operative procedures ; sometimes the wound has healed and tetanus has followed operation for the removal of foreign bodies in the tissues. Again the disease tends to assume the type seen in some animals, the muscles in the neighbourhood of the wound being first affected ; local hardness and stiffness, pain, and exaggeration of local reflexes have thus often been the first, and sometimes the only, clinical phenomena. Such cases of tetanus are also apparently more amenable to treatment with anti-tetanus serum.

The pathological changes found *post mortem* are not striking. There may be hæmorrhages in the muscles, which have been the subject of the spasms. These are probably due to mechanical causes. It is in the nervous system that we naturally look for

the most important lesions. Here there is ordinarily a general redness of the grey matter, and the most striking feature is the occurrence of irregular patches of slight congestion which are not limited particularly to grey or white matter, or to any tract of the latter. These patches are usually best marked in the grey matter of the medulla and pons. Microscopically there is little of a definite nature to be found. There is congestion, and there may be minute hæmorrhages in the areas noted by the naked eye. The ganglion cells may show appearances which have been regarded as degenerative in nature, and similar changes have been described in the white matter. The only marked feature is thus a vascular disturbance in the central nervous system, with a possible tendency to degeneration in its specialised cells. Both of these conditions are probably due to the action of the toxins of the bacillus. In the other organs of the body there are no constant changes.

(*b*) *The Artificially-produced Disease.*—The disease can be communicated to animals by any of the usual methods of inoculation, but does not arise in animals fed with bacilli, whether these contain spores or not. Kitasato found that pure cultures, injected subcutaneously or intravenously, caused death in mice, rats, guinea-pigs, and rabbits. In mice, symptoms appear in a day, and death occurs in two or three days, after inoculation with a loopful of a bouillon culture. The other animals mentioned require larger doses, and death does not occur so rapidly. Usually in animals injected subcutaneously the spasms begin in the limb nearest the point of inoculation. In the case of intravenous inoculation the spasms begin in the extensor muscles of the trunk, as in the natural disease in man. After death there is found slight hyperæmia without pus formation, at the seat of inoculation. The bacilli diminish in number, and may be absent at the time of death. The organs generally show little change.

Kitasato stated that in his earlier experiments the quantity of culture medium injected along with the bacilli already contained enough of the poisonous bodies formed by the bacilli to cause death. The symptoms came on sooner than by the improved method mentioned below, and were, therefore, due to the toxins already present. In his subsequent work, therefore, he employed splinters of wood soaked in cultures in which spores were present, and subsequently subjected for one hour to a temperature of 80° C. The latter treatment not only killed all the vegetative forms of the organism, but, as we shall see, was sufficient to destroy the activity of the toxins. When such

splinters are introduced subcutaneously, death results by the development of the spores which they carry. In this way he completed the proof that the bacilli by themselves can form toxins in the body and produce the disease. Further, if a small quantity of garden earth be placed under the skin of a mouse, death from tetanus takes place in a great many cases. By such experiments, supplemented by the culture experiments mentioned, the natural habitats of the B. tetani, as given above, have become known.

The Toxins of the Tetanus Bacillus.—The toxic properties of bacterium-free filtrates of pure cultures of the B. tetani were investigated in 1891 by Kitasato. This observer found that when the filtrate, in certain doses, was injected subcutaneously into mice, tetanic spasms developed, first in muscles contiguous to the site of inoculation, and later all over the body. Death resulted. He found that guinea-pigs were more susceptible than mice, and rabbits less so. In order that a strongly toxic bouillon be produced, it must originally have been either neutral or slightly alkaline. Kitasato further found that the toxin was easily injured by heat. Exposure for a few minutes at 65° C. destroyed it. It was also destroyed by twenty minutes' exposure at 60° C., and by one and a half hours' at 55° C. Drying had no effect. It was, however, destroyed by various chemicals such as pyrogallol, and also by sunlight.

To prepare the toxin, freshly made veal bouillon not too long autoclaved should be used, and a massive inoculation, preferably from a fluid culture, practised. Individual strains of the bacillus differ in their capacity for producing toxin. The culture must be incubated under anaerobic conditions, and the maximum toxicity is developed in from ten to fifteen days. Behring pointed out that after the filtration of cultures containing toxin, the latter may very rapidly lose its power, and in a few days may only possess 100th of its original toxicity. This is due to such factors as temperature and light, and especially to the action of oxygen. Toxins should thus have a layer of toluol floated on the surface and be kept in a cool, dark place. The effect of harmful agents on the crude toxin is apparently to cause a degeneration of the true toxin so as to form what it is convenient at present to call *toxoids*, similar to those produced in the case of diphtheria toxin, and it is also true here that the toxoids while losing their toxicity may still retain their power of producing immunity against the potent toxin. Further, altogether apart from the occurrence side by side in the crude toxin of strong and weak poisons, it has been shown that such crude toxin may contain different varieties of toxic substances. Ehrlich showed that besides the predominant spasm-producing toxin (called by him *tetanospasmin*), there often exists in crude toxin a poison capable of producing the solution of certain red blood corpuscles. This hæmolytic agent he called *tetanolysin*.

It does not occur in all samples of crude tetanus toxin, nor is it found when a bouillon culture of the bacillus is filtered through porcelain. To obtain it, the fresh culture must be treated by ammonium sulphate, as described in the method of obtaining concentrated toxins (p. 167). Tetanolysin also has the power of originating an antitoxin, so that certain anti-tetanic sera can protect red blood corpuscles against its action. Madsen, studying the interactions of this anti-tetanolysin with the tetanolysin, showed that phenomena can be demonstrated similar to those noted by Ehrlich as occurring with diphtheria toxin, which the latter interpreted as indicating the presence of degenerated toxins (toxoids) in the crude poison.

As with other members of the group, nothing is known of the nature of tetanus toxin. Uschinsky has found that the tetanus bacillus can produce its toxin when growing in a fluid containing no protein matter. The toxin may thus be formed independently of the breaking up of the proteins on which the bacillus may be living, though the latter has a digestive action on proteins. There is evidence that peptic digestion and toxin formation are due to different vital processes on the part of the tetanus bacillus.

The toxin is one of the most powerful poisons known. Even with an impure " toxalbumin " consisting practically of an alcoholic precipitate from filtered cultures in bouillon, Brieger found that the fatal dose for a mouse was 0·0005 of a milligram. If the susceptibility of man be the same as that of a mouse, the fatal dose for an average adult would have been 0·23 of a milligram. Within recent years such attempts to isolate tetanus toxins in a pure condition have practically been abandoned, and attention has been turned to the investigation of the physiological effects either of the crude toxin present in filtered ordinary bouillon cultures grown under anaerobic conditions, or of the precipitate produced from the same by ammonium sulphate (cf. p. 167). Animals differ very much in their susceptibilities to the action of tetanus toxin. According to v. Lingelsheim, if the minimal lethal dose per gram weight for a horse be taken as unity, that for the guinea-pig would be 6 times the amount, the mouse 12, the goat 24, the dog about 500, the rabbit 1800, the cat 6000, the goose 12,000, the pigeon 48,000, and the hen 360,000.

A striking feature of the action of tetanus toxin is the occurrence of an incubation period between the introduction of the toxin into an animal's body and the appearance of symptoms. This varies according to the species of animal employed, the path of infection, and the dose given. In the guinea-pig it is from thirteen to eighteen hours, in the horse five days, and the incubation is shorter when the poison is introduced into a vein than when injected subcutaneously. In man the period between the receiving of an injury and the appearance of tetanic symptoms is usually from two to fourteen days, but this period may be lengthened, and the bacilli may remain a considerable time shut up in a wound before producing effects. The longer

the incubation period, the more favourable is the prognosis, and in chronic cases spontaneous recovery is not uncommon.

With regard to the action of the toxin it has been shown that when a dose is introduced directly into the blood stream it disappears rapidly from the circulation. This, no doubt, is due partly to its passing into the lymph, but mainly to its combining with certain tissues, especially the central nervous system; and the latter has been found to fix the toxin *in vitro*. The local muscular spasm which is the initial phenomenon in ascending tetanus is due to the action of the toxin on the related segments of the spinal cord. The toxin reaches the cord by passing up the motor nerves (Marie and Morax; Meyer and Ransom; Permin and others). This has been demonstrated by taking advantage of the fact that tetanus toxin when injected into a muscle soon reaches and enters the nerve supplying the muscle, and in that situation is practically insusceptible to the neutralising action of antitoxin contained in the blood and lymph. A rabbit received an intravenous injection of antitoxin; then a small dose of tetanus toxin was injected into the muscles of a hind leg. The result was that local tetanus developed in the inoculated limb and nowhere else. If, under similar conditions, the sciatic nerve was first cut below the gluteus maximus and then toxin was injected into the peripheral portion no tetanus occurred. Again, if antitoxin was injected into the sciatic nerve and then toxin was immediately injected into the corresponding limb, tetanus did not develop in it. The latent period which follows inoculation with the toxin is also greatly shortened, although not abolished, by direct injection of toxin into the spinal cord. The tetanic spasms start in the muscles corresponding to the site of inoculation, and then spread to other muscles as the toxin diffuses in the cord. Tetanus toxin also reaches the blood stream from a local infection, the amount present in the blood being usually at a maximum before and at the commencement of the outbreak of tetanic symptoms. Toxin has also been demonstrated in the cerebro-spinal fluid in human cases. The general muscular involvement of descending tetanus, as seen in man or the horse, depends on the diffused action on the nervous system of toxin disseminated by the blood and lymph. Even in small animals, when the injection of toxin is made into a non-muscular region, general descending tetanus follows. Whether the toxin passes to the nerve centres directly from the blood stream, or whether it reaches them only by way of the muscular nerves after being taken up from the lymph by the nerve endings, is a point which has not been satisfactorily determined. The early

development of spasm in the masseters and the neck most probably depends on the weak action of the antagonising muscles which renders the spasm apparent in these situations.

The precise mode of spread of toxin in the nerves has not been decided. According to one view the axis cylinders are the conductors, and in support of this is the fact that a rabbit very highly immunised to tetanus toxin may develop fatal tetanus as the result of an injection of toxin into the sciatic nerve, although several drops of its blood or lymph contain more than sufficient antitoxin to neutralise the dose of toxin injected. On the other hand, it has been held that the toxin ascends in the neural lymphatics. It is only the motor nerves which conduct the toxin to the motor cells of the cord. An injection of toxin into the posterior nerve roots between the ganglion and the spinal cord does not produce tetanus, but causes severe spasmodic pains (tetanus dolorosus). In addition to the action on the cord the toxin affects the brain. This has been most clearly shown by injecting toxin into the vitreous humour of rabbits which had previously received an intravenous injection of antitoxin. The animals developed general convulsions with practically no tonic spasm (Permin).

As regards the nature of the nervous lesions it appears that the anterior cornual cells of the cord are irritated by the toxin, thus giving rise to increased muscular tonus. According to Sherrington's investigations, disturbance of reciprocal innervation also occurs. Thus stimulation of an area of the cerebral cortex, which in a healthy animal causes opening of the mouth—due to contraction of the depressors of the jaw, accompanied by the relaxation of the elevators—in a tetanic animal leads to trismus, since the normal inhibition of the closing muscles, which form the more powerful group, is now converted into stimulation.

Immunity against Tetanus.—Anti-Tetanic Serum.—The artificial immunisation of animals against tetanus received much attention, especially from Behring and Kitasato in Germany, and Tizzoni and Cattani in Italy. The former observers found that a degree of immunity could be conferred by the injection of very small and progressively increasing doses of the tetanus toxin. Subsequent work has shown that the richer a crude toxin is in modifications of the true toxin (toxoids), the more useful it is for immunisation procedures. In fact it is doubtful if small animals can be immunised at all by fresh filtrates. In some cases the injection of non-lethal doses instead of commencing an immunity actually increases the susceptibility of the animal, and this may

be related to the development of supersensitiveness to proteins generally (see " Anaphylaxis," p. 223).

More successful in producing immunity are the methods of accompanying the early injections of crude toxin with the subcutaneous introduction of small doses of iodine terchloride, or of using toxin which has been acted on by iodine terchloride or by iodine itself. Living cultures attenuated in various ways, *e.g.* by heat, have also been used. By any of these methods susceptible animals can be made to acquire great immunity against large doses of tetanus toxin, and also against living bacilli. Immunity thus acquired remains in existence for a long time. The serum of such immune animals possesses the capacity of protecting animals susceptible to the disease against a subsequent injection of a fatal dose of tetanus bacilli or toxin. Further, if injected subsequently to infection, the serum can in certain cases prevent a fatal result, even when symptoms have begun to appear. The degree of success attained depends, however, on the shortness of the time which has elapsed between the injection of the bacilli or of toxin and the injection of the serum. In animals where symptoms have fully manifested themselves only a small proportion of cases can be saved. As with other antitoxins, there is no evidence that the anti-tetanic serum has any detrimental effect on the bacilli. It only neutralises the effects of the toxin. The standardisation of the anti-tetanic serum is of the highest importance. Behring recommended that for protecting animals a serum should be obtained of which 1 gram will protect 1,000,000 grams weight of mice against the minimum fatal dose of the bacillus or toxin. A mouse weighing 20 grams would thus require 0·00002 gram of the serum to protect it against the minimum lethal dose. On the injection of such a serum subsequent to infection, if symptoms have begun to appear, 1000 times this dose would be necessary ; a few hours later 10,000 times, and so on.

Agglutination.—Cultures of tetanus bacilli which have been washed and heated to deprive them of toxin, when injected into rabbits, lead to the production of agglutinins. By this means Tulloch has shown that the organisms fall into five serological groups. It is to be noted, however, that there is no difference in the toxins of the different groups as an antitoxin developed by one type neutralises the toxin from all of the four.

The Therapeutic Application of Tetanus Antitoxin.—As the results of his experiments, Behring aimed at obtaining a curative effect in the natural disease occurring in man. For this purpose he immunised large animals such as the horse, the sheep, and the goat. It is found that the greater the degree of the natural susceptibility of an animal to tetanus, the easier is it to obtain a serum of a high anti-tetanic potency. The horse is, therefore, the most suitable animal and is usually employed.

The serum is now standardised by a method similar to that set up for diphtheria antitoxin (see Chapter VI.), and its strength

is reckoned in terms of similar units. In this country the unit ordinarily used is that determined by the method practised in the U.S.A., and the sera contain from 150 to 800 units per c.c. Sera maintain their potency for a considerable period, but a serum more than a year old should not be used unless it has been subjected to fresh standardisation. Sera should always be stored in a dark and cool place but not frozen.

The essential factors for the success of serum therapy are, first, that there should not be an hour of unnecessary delay in commencing treatment after a case is seen, and secondly, that the antitoxin should be given in proper amount. This has been demonstrated clearly by experiment. MacConkey determined the amount of antitoxin sufficient to save the life of a guinea-pig when injected under the skin simultaneously, but at another site, with a dose of toxin just sufficient to kill an untreated animal; he then found that one-tenth of this amount of serum sufficed to neutralise 100 fatal doses of toxin when toxin and antitoxin were mixed together *in vitro* prior to injection; and two thousand times as much serum was required to save the life of the animal when injected twenty-four hours after the toxin, *i.e.* at a time when symptoms of tetanus had already appeared. As has been seen, the toxin rapidly disappears from the blood stream; but even after this has occurred, antitoxin is still up to a point capable of exerting a curative effect. Whether the antitoxin can act by causing dissociation of toxin already fixed to vulnerable cells is, however, uncertain. The serum may be given subcutaneously, intramuscularly, intravenously, or intrathecally by lumbar puncture. In the first two methods absorption is relatively slow—in the last elimination is relatively rapid. But by the intrathecal method the serum gains rapid access to the grey matter of the cord on which the toxin is exerting its specific action. Sherrington treated monkeys inoculated with tetanus toxin after the development of symptoms either by an intrathecal or an intramuscular injection of antitoxin and showed the great superiority of the former method under the conditions studied. The earlier injections ought therefore to be given both intrathecally and also intravenously, the latter route being chosen because antitoxin can be quickly administered, and as Henderson Smith has shown, a high concentration of antitoxin in the body fluids is maintained for a considerable time; the neutralisation of toxin passing out from a focus of infection is thus facilitated. Later, injections should be given by intramuscular and subcutaneous routes, the principle being that, as the antitoxin first given is eliminated, its place is taken by the more slowly absorbed and therefore more grad-

ually eliminated moieties. Andrewes has recommended the initial injection intrathecally of 20,000 to 30,000 units (in the form of concentrated antitoxin), and repeated doses may be given for several days; 10,000 to 15,000 units are given intravenously along with the first intrathecal dose, and a similar amount is injected subcutaneously three or four days later.

The results of the therapeutic use of antitoxin in tetanus have not been so good as in the corresponding case of diphtheria. The great difficulty is that an infection is not suspected till the tetanus bacilli have already begun to manifest their gravest effects, by which time the nerve cells may have been damaged irreparably.

The Prophylactic Use of Tetanus Antitoxin.—The practice of giving antitoxin prophylactically in every case of a ragged, unhealthy-looking wound, especially when contaminated with soil, has been advocated. The principle has, for a considerable time, been applied in connection with the injuries contracted during the Independence Day celebrations in America, of which tetanus is a not uncommon sequel; a very definite fall in the death-rate has been thereby effected. It was during the late war, however, that the success of prophylaxis was established. During the early months, in the fighting on the Continent, tetanus was rife—its incidence in the wounded brought to Britain being about sixteen per thousand. After the autumn of 1914 prophylactic injections of antitoxin were given to every wounded man, with the result that the corresponding incidence was reduced to two per thousand. The initial dose is 500 units administered subcutaneously, and, as passive immunity is of relatively short duration, this dose should be repeated at seven-day intervals till four doses have been given. Further, when at later periods operative interference, even with healed wounds, is necessary, a similar dose should be given, either subcutaneously forty-eight hours, or intramuscularly twelve hours, previous to the operation.

Attention has already been directed to the effects of the prophylactic use of antitoxin in modifying the clinical type of the disease.

Methods of Examination in a Case of Tetanus.—The occurrence of " drumstick " bacilli in stained smears of the discharge from a wound or in necrotic tissue excised from its margin is suggestive, but other organisms with round terminal spores may occur (M'Intosh). Again, the tetanus bacillus in the non-sporing condition is not characteristic in appearance. A much more certain

method of diagnosis is the production of tetanus by a subcutaneous injection into mice of pathological material either directly or, preferably, as a filtrate from fluid anaerobic cultures. Or the organisms may be isolated in the pure state by culturing secretions or excised tissues from the wound by Fildes' method (p. 527). It must be remembered, however, that these procedures take too long a time to afford a useful guide for administration of antitoxin. The history of a contaminated wound should be the indication for treatment.

Bacillus Botulinus

The term " meat-poisoning " embraces a number of conditions produced by different agents, and the bacilli related to one class of case have already been discussed (p. 425). Another group was shown by van Ermengem in 1896 to be caused by an anaerobic bacillus to which he gave the name *Bacillus botulinus* (*Clostridium botulinum*). He cultivated the organism from a sample of ham, the ingestion of which in the raw condition had produced a number of cases of poisoning, some of them followed by fatal result. It may be noted that the ham did not show signs of decomposition in the ordinary sense. The symptoms in these cases closely corresponded with those occurring in the so-called " sausage-poisoning." Such cases form a fairly well-defined group, the symptoms in which are chiefly referable to an action on the medulla, and, as will be detailed below, similar symptoms have been experimentally produced by means of the bacillus mentioned or its toxins. The chief symptoms of botulismus are disordered secretion in the mouth and nose, more or less marked ophthalmoplegia, externa and interna (dilated pupil, ptosis, etc.), dysphagia, and sometimes aphagia with aphonia, marked constipation and retention of urine, and in fatal cases interference with the cardiac and respiratory centres. Along with these there is practically no fever and no interference with the intellectual faculties. The symptoms commence in the human subject usually twelve to twenty-four hours after ingestion of the poison. During recent years there have been a fairly large number of cases of botulismus in America, and the bacillus has been cultivated or its toxin identified in a considerable proportion of these. The source has been mainly tinned meats, fruits, and vegetables, which have been contaminated by the bacillus and in which it has grown. B. botulinus has been cultivated in certain instances from the intestines of animals, and it has been found to have a wide distribution in America in soils, both arable and virgin ; the method of detection employed has been to make anaerobic cultures in fluid media and then to

test the toxicity of the filtrate, this being controlled by the use of the specific antitoxin.

Microscopical and Cultural Characters.—The organism is a bacillus of considerable size, measuring 4 to 9 μ in length and 0·9 to 1·2 μ in thickness; it has somewhat rounded ends and sometimes is seen in a spindle form. It is often arranged in pairs, sometimes in short threads. Under certain conditions it forms spores which are oval in shape, terminal or sub-terminal in position, and only a little thicker than the bacilli. The spores have greater resistance to heat than was at first supposed, and may in certain circumstances withstand moist heat of 100° C. for more than an hour. The B. botulinus is a motile organism, and has 4 to 8 lateral flagella of wavy form. It stains readily with the ordinary dyes, and is Gram-positive, though care must be employed in decolorising.

The B. botulinus can be readily cultivated on the ordinary media, but only under strictly anaerobic conditions. Cultures in glucose agar resemble those of certain other anaerobes; there is abundant development of gas, and the medium is split up in various directions. In glucose gelatin a whitish line of growth forms with lateral offshoots, but liquefaction with abundant gas formation soon occurs. In gelatin plates the colonies after four to six days are somewhat characteristic; they appear to the naked eye as small semi-transparent spheres, and these on examination under a low power of the microscope have a yellowish-brown colour and are seen to be composed of granules which show a streaming movement, especially at the periphery. The cultures have a rancid, though not foul, odour, due chiefly to the development of butyric acid. The bacillus does not liquefy coagulated serum, and M'Intosh places it in the non-proteolytic group. He finds that it ferments glucose, with evolution of gas, and, to a less extent, maltose, lactose, glycerin, and starch. The optimum temperature is below that of the body, namely, between 20° and 30° C.; at the body temperature growth is slower and less abundant and spore formation does not occur. A point of importance is that at body temperature little toxin is produced (Dickson).

Pathogenic Effects.—Like the B. tetani, the B. botulinus has little power of flourishing in the tissues, whereas it produces a very powerful filtrable toxin. When a fairly large dose of toxin is injected subcutaneously in a guinea-pig, symptoms chiefly of paralytic nature may appear within about six hours and death follow within twenty-four hours. When the dose is sub-lethal a somewhat chronic condition may result in which local paralysis

forms a striking feature. The characteristic effects can also be produced by oral administration of the filtered toxin, though in this case the dose requires to be larger. As in the case of the tetanus poison, the potency of the toxin is remarkable, the fatal dose by subcutaneous injection for a guinea-pig of 250 grams weight being in some instances 0·0001 c.c. of the filtered toxin or even less. When massive doses of toxin-free spores are given by the alimentary canal it has been found that multiplication of the organisms and toxin production may follow in certain cases, but there is no evidence that the disease in the human subject is produced in any other way than by the ingestion of previously formed toxin in the contaminated food. The presence of the toxin in infected ham was first shown by van Ermengem by inoculation with a watery extract, and a similar result has been repeatedly obtained by others in the case of other articles of diet which have produced the disease. The organism has been recovered from the organs of those who have died of the disease, but it is only after or immediately before death that a few bacilli may enter the tissues.

The properties of the botulinus toxin have been investigated, and have been found to correspond closely, as regards relative instability, conditions of precipitation, combination with sensitive cells (*i.e.* of brain and cord), etc., with the toxins of diphtheria and tetanus. An antitoxin was prepared by Kempner by the usual methods, and was shown not only to have a neutralising property, but to have considerable therapeutical value when administered some hours after the toxin. These results have been fully confirmed. The subject was studied by Leuchs, and he found that the combination toxin-antitoxin can be split up by the action of acids and the two components recovered, just as Morgenroth showed to occur in the case of diphtheria (p. 193). As was shown by Dickson and Howitt, there are two types of the B. botulinus (known as A and B) which differ in the toxins which they produce ; the antitoxin to toxin A has no effect on toxin B, and vice versa. This is, of course, an important matter in relation to treatment, and bivalent antitoxin has now been introduced in America. It has been found that in the eastern states most of the outbreaks have been due to type A, whilst in the western type B has been more frequently found. A similar result has been obtained with regard to the distribution of the two types in the soil in these districts. It has been found that fowls are very susceptible to the toxin of type A, dying within twenty-four hours after oral administration, whereas they are resistant to the toxin

of type B. Botulismus occurs in natural conditions in these animals and is known as "limber-neck," and in this affection the bacillus of type A has been found to be the causal organism (Graham and Schwarze). The condition of the nerve cells in experimental poisoning with the botulinus toxin was investigated independently by Marinesco and by Kempner and Pollack, and these observers agree as to the occurrence of marked degenerative changes, especially in the motor cells in the spinal cord and medulla. On the other hand, Dickson and others have found that the toxin has an important action on the vascular intima, leading to thrombosis in the small vessels; in this way the nerve cells are damaged or necrosed.

Anaerobes in Infected Wounds

It may be said that practically all such anaerobes come from the soil and that their original source is chiefly animal fæces. All cultivated soils are accordingly rich in such organisms. In the case of lacerated wounds contaminated by soil, and especially gunshot wounds, we have thus two main factors, the presence of damaged or necrosed tissues, and infection by various anaerobes of intestinal origin, though there are also some aerobes present from the same and other sources. At an early stage the number and variety of organisms, many of them spore-bearing, form a very striking feature. As reactive processes, exudation, leucocyte emigration, etc., come into play, we find in favourable cases that the anaerobes gradually diminish, while the aerobes continue to flourish, though in deep clefts and in necrosed tissue the former may persist for a long time. This change in the flora becomes more marked as suppuration becomes established and progresses; the ordinary pyogenic organisms multiply at the expense of the various bacilli, enterococci, etc., till ultimately they are the chief organisms present. So far as serious complications are concerned, we may say that in the early stages these are chiefly due to the anaerobes, and in the latter stages to streptococci and, to a less degree, to staphylococci. We have here to deal with the anaerobes, and these have the following effects: (*a*) poisoning by toxins, the outstanding example being of course the B. tetani; (*b*) invasion of the tissues, the production of spreading œdema, necrosis, and gaseous emphysema—generally comprised under the term "gas gangrene"; and (*c*) merely local inflammatory and putrefactive changes. The B. tetani has already been treated of, and it has been shown that it has no more infective or invasive properties than other

saprophytic anaerobes. The number of anaerobes separated from wounds during the war was large, and with regard to them two general statements may be made. In the first place, only a few have been shown to cause by themselves definite spreading infections. Of these it is generally accepted that the *B. welchii* is by far the most important, next comes the '*vibrion septique*,' and then probably the *B. œdematiens*. In the second place, the organisms which sometimes cause these serious results may be present in wounds from which no complications arise. There must accordingly be favouring conditions in certain cases which lead to these grave and often fatal results. This, of course, holds with regard to bacterial infections in general, but it is especially well exemplified in the lesions in question. As possible determining influences, we might mention the degree of the injury, the dose, and possibly the virulence, of the invading organism, and the adjuvant effect of other organisms. And as shown by Gye and Cramer (p. 153), the presence of soluble calcium salts has a very important effect in rupturing the defences and leading to infection by anaerobes. In the case of gas gangrene produced by the B. welchii, infection of lacerated muscle has been shown to be an extremely important factor in its origin and spread (*vide infra*).

This group of organisms (genus *Clostridium*) may be said to have the following characters. They are, on the whole, fairly large bacilli, easily stained and Gram-positive, though the occurrence of Gram-negative forms is fairly common in older cultures. Spore-formation is the rule; the spores, which are rounded or oval, have a thickness exceeding that of the bacillus, sometimes markedly so, and may be terminal, subterminal, or central in position. In a given species the position of the spore may vary somewhat, but in the case of some, *e.g.* the B. tetani, the spore is always terminal. The majority possess numerous lateral flagella, and many are actively motile; a few, *e.g.* the B. welchii, are non-motile. The earlier means of differentiation depended on morphological and cultural features in a few media, and on pathogenic effects. All these factors, however, vary somewhat. More recently the physical and chemical changes produced in various definite media have been added as a means of distinguishing them. The important work of M. Robertson, Henry, Wolf and Harris, and M'Intosh may be mentioned in this connection, and to their publications we are indebted for many of the facts stated below. The appearances of superficial and deep colonies have also been found of service. The result of biological inquiries has been to divide the organisms according to their metabolic activities into two main groups, namely, (1) the

saccharolytic or *non-proteolytic* and (2) the *proteolytic*. This distinction must be taken in a broad sense, as the proteolytic members have an action on some sugars. Variations are met with in the rapidity of the fermentation and also in the products which are ultimately formed. The recognition of these two main groups is of importance also from the pathological point of view, as the chief organisms which produce spreading lesions belong to the saccharolytic group. In fact there is often a "saccharolytic stage" of advancing infection, followed by a "proteolytic stage" of putrefaction. We shall give the chief characters of the most fully studied of these organisms, dealing first with the non-proteolytic, which are the most important.

Bacillus Welchii (B. Aerogenes Encapsulatus)

This bacillus was first described by Welch and Nuttall in 1892, who showed that it was the cause of the extensive gaseous development which sometimes occurs in the organs *post mortem*, resulting in the formation of rounded gas cavities. It is now recognised that it is identical with an organism cultivated later by E. Fraenkel and called by him the *B. phlegmonis emphysematosæ*. The same bacillus was described by Veillon and Zuber, who gave it the name *B. perfringens*. During the war it came into great prominence, as it was proved to be by far the most important agent in the production of gas gangrene. We shall speak of it as the B. welchii (*Clostridium welchii*).

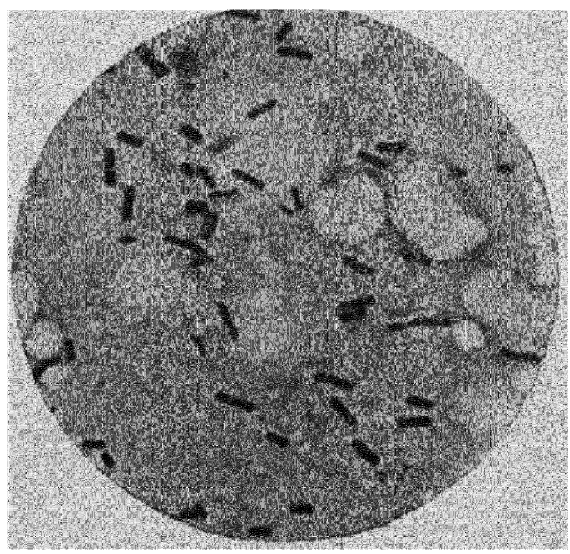


Fig. 147.—Film taken from margin of spreading gas gangrene, showing numerous examples of B. welchii (pure).[1] Gram's stain. ×1000.

Microscopical Characters. — As seen in the serous fluid in a case of spreading gas gangrene, it is a comparatively large bacillus, measuring usually 4–6 μ in length (Figs. 147, 148) and

[1] We are indebted to Major J. W. M'Nee, R.A.M.C., for the preparation from which Figs. 147, 148, 149, and 154 were made.

relatively stout; but the thickness varies somewhat. Its ends are somewhat rounded, though those of some of the shorter forms are almost square. In cultures it is rather pleomorphous, and in sugar-free media there is a tendency to form filaments (Henry); again, short, almost coccus-like forms, may be met with. It is readily stained with the basic dyes, and is Gram-positive, though in older cultures Gram-negative forms occur. In the tissue fluids it usually has a distinct and fairly broad capsule—hence the original name; sometimes, however, no capsule is seen. In ordinary media, again, no capsule is seen, but in serum media it can be demonstrated by special methods (*cf.* pneumococcus). The organism is non-motile, and no flagella have been demonstrated.

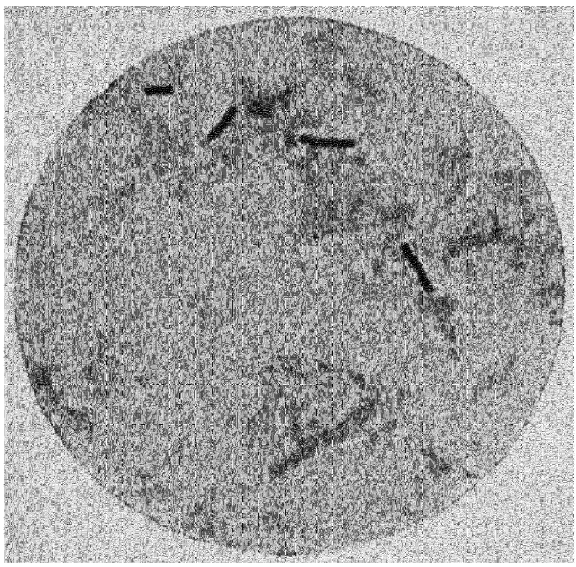


FIG. 148.—Film from necrosed muscle in gas gangrene, showing a few B. welchii with remains of muscle fibres.
Gram's stain. ×1000.

In the spreading area of the disease no spores are found, though they have been described in the later stages when the bacillus is associated with other organisms. At first it was believed not to form spores, but, as was first shown by Dunham, spores are produced in serum media; they are oval and fairly large, usually subterminal, occasionally central. In ordinary

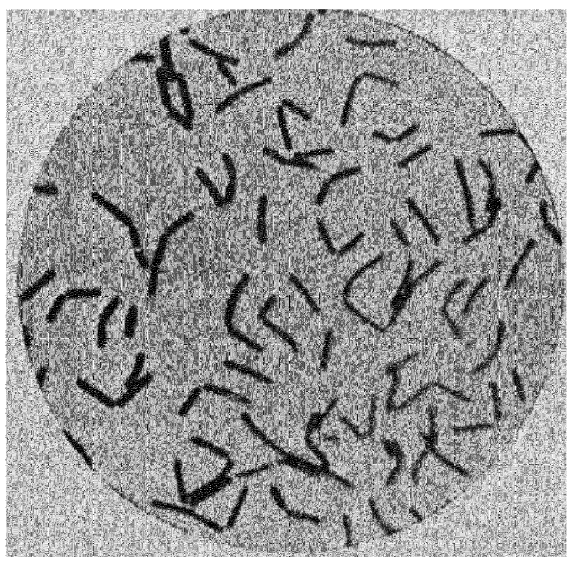


FIG. 149.—Film from a pure culture of B. welchii.

media, however, and in the presence of a trace of sugar, no spores are formed.

Cultivation.—The B. welchii can be readily grown on various media, but only under strict anaerobic conditions. It flourishes best at the temperature of the body, but grows also at room temperature. On serum agar the superficial colonies are circular in form, moist in appearance, with smooth margins, there being no radiate outgrowth or downgrowth; the deep colonies are usually oval or lenticular in form, with sharp outline. It produces no liquefaction either in gelatin or in solidified serum. In milk the characters of the growth are of importance. It grows rapidly and leads to production of coagulation of the medium, the clot becomes broken up by gas bubbles—the so-called "stormy reaction"—and ultimately forms irregular tough masses bathed in comparatively clear whey. There is no digestion of the casein even after a long time. The culture has an odour of butyric acid. These effects in milk are practically the same as those described by Klein in the case of his B. enteritidis sporogenes, and the two organisms concerned in them are probably the same. In cooked meat medium the B. welchii produces a pink colour with considerable amount of gas; there is a sour smell, but no putrid odour or blackening of the medium. It produces acid and gas from glucose, maltose, lactose, saccharose, and starch, and also from glycerine; inulin is sometimes fermented, sometimes not. Fermentation of these substances takes place with great rapidity, but the acid formed has a markedly deterrent action on the growth, and soon leads to its cessation. The B. welchii has thus very active saccharolytic action, whereas its digestive effect on proteins is almost nil, and these properties will be found to have an important bearing on its pathogenic effects.

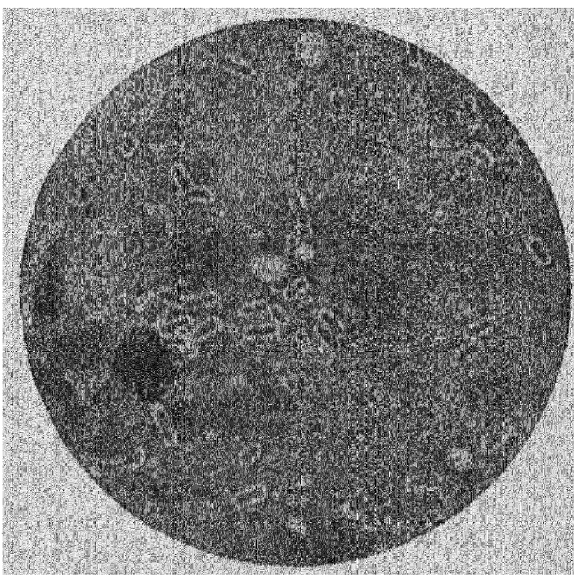


FIG. 150.—Bacillus welchii, showing capsules; film preparation from bone-marrow in a case where gas cavities were present in the organs. ×1000.

Pathogenic Effects.—In addition to invading the blood stream about the time of death, and giving rise to gas cavities in the organs, the B. welchii has been found in various emphysematous and gangrenous conditions in civil life, the infection starting in connection with the alimentary canal. It is also, as has been indicated, by far the most important cause of gas gangrene from war wounds. In this affection it is now recognised that the starting-point is usually some laceration of muscle, which has become contaminated with soil containing the bacillus. The spread of the disease is often remarkable, as cases have been recorded in which extensive emphysematous swelling with gangrene of a limb has occurred with a fatal result, well within twenty-four hours. In some cases the affection may be confined to individual muscles, and resection of these has been carried out, sometimes with success. Within a muscle, the necrotic change may affect individual fibres, leaving others in relation to them unaffected. The stages as described by M'Nee and Shaw Dunn are as follows. The bacilli spread with great rapidity along the interstitial tissue of the muscle, and may be found beyond the actual site of gangrene. They are present often in very large numbers and in practically pure culture. The fibres thus surrounded become somewhat swollen, altered in staining reaction, and separated from the interstitial tissue by a zone of serous fluid, poor in protein. The fibres then become completely necrosed, the sarcolemma nuclei losing their staining reaction, and about this time the fluid within the sarcolemma comes to contain the bacilli in large numbers. There is then evolution of gas, chiefly from the muscle carbohydrates, and the muscle substance becomes broken up and disintegrated, though the transverse striation may persist for a considerable time (Fig. 148). Finally the dead muscle may be invaded by other organisms, and become putrid and softened. Along with these changes in the muscle there occur œdema and emphysema in the interstitial and subcutaneous connective tissue, while the skin shows various kinds of discoloration, and the affected part is swollen, tense, and gives crackling on palpation.

To the naked eye the affected muscle is at first swollen and pale and has lost its elasticity; it soon assumes a brownish-red colour, is beset with gas bubbles and is putty-like in consistence, while later it becomes brownish-yellow, greenish, or dark red. It must be noted, however, that the bacillus does not cause the ordinary changes of putrefaction.

Wright found that locally there is a fall in the anti-tryptic

action of the serum along with increased acidity, and he considered that the co-existence of these factors favours the growth of the bacilli and leads to their rapid spread. The spread of the bacilli is attended by practically no leucocyte reaction, unless when the spread is becoming arrested or when it takes place around other organisms. The growth of the bacilli is essentially local, but they may enter the blood shortly before death, when they have been found in a certain proportion of cases. Instances have also been recorded in which they have settled in other parts of the body and produced lesions there—the so-called metastatic gas gangrene.

Experimental Inoculation.—The virulence of the B. welchii varies considerably. In the case of many strains fatal gas gangrene may be produced in guinea-pigs by injection of cultures in fluid media, the bacterial invasion being favoured by the toxin present. The bacilli by themselves, for example when obtained from a surface growth on a solid medium, do not produce infection, but Gye and Cramer showed that when an ionisable calcium salt is injected, the bacilli rapidly invade the tissues affected by the salt and cause a rapidly fatal infection. In the experimental disease the bacilli are abundant locally, but only a few are present in the blood stream. When the dose is sublethal a local gangrene may occur, with subsequent separation of the dead tissue ; thereafter healing may rapidly follow. Intramuscular injection is the most effective method, especially in the rabbit, which is more resistant than the guinea-pig. The pigeon is found to be the most susceptible of the animals hitherto tested, the lethal dose being only a fraction of that for the guinea-pig. Injection into the pectoral muscle of a pigeon causes lesions in the muscle closely resembling those in gas gangrene in man, and death follows very rapidly, sometimes within a few hours. Most observers have found filtered cultures to be practically non-toxic, but Bull and Pritchett have succeeded in obtaining a true exotoxin. The medium used by them was plain meat bouillon containing fragments of sterile skeletal muscle of the pigeon or rabbit. After inoculation the medium is incubated under anaerobic conditions at 37° C. for twenty-four hours, and is then filtered through a Berkefeld N candle. The filtrate was found to be highly toxic for all the animals mentioned above, and gave rise to local lesions closely resembling those caused by the bacilli themselves. In addition to having a local necrotic effect on muscle, the toxin, or a moiety of it, is actively hæmolytic, and leads to a massive destruction of red corpuscles when injected intravenously. Bull and

Pritchett believe, accordingly, that death from gas gangrene is due to a true toxæmia, and not to the production of acid in the tissues, as has been supposed by some. By means of injecting carefully graduated doses of the toxin, they produced an active immunity, and the serum of the treated animals possessed antitoxic properties. The antitoxin neutralises all the effects of the toxin in multiple proportions, and is protective and curative against infection with the bacillus in the pigeon. These results were obtained towards the end of the war, and so far as we know the therapeutic effect of the antitoxin in the human subject was not tested on a large scale.

Bacillus fallax.—This organism was separated by Weinberg and Séguin, and the name was given by them on account of its resembling the B. welchii. It is smaller than the latter organism, being both somewhat thinner and shorter. It is Gram-positive, and in cultures forms spores which are usually subterminal in position. It possesses lateral flagella and is feebly motile. The growths resemble those of B. welchii, but the young surface colonies are more transparent and the older ones have a more irregular margin. The action on milk is much less marked, the formation of clot and gas usually occurring only after several days; the action on sugars also is feebler and more restricted. It has no digestive effect on casein or on coagulated serum. It thus may be described as a non-proteolytic bacillus with somewhat weak saccharolytic action. Recent cultures produce a gelatinous œdema on injection into a guinea-pig; they, however, soon lose their virulence.

Vibrion Septique (Pasteur), Bacillus of Malignant Œdema (Koch)

The *vibrion septique* was first discovered by Pasteur in putrefying carcasses. He described its characters, distinguishing it from the B. anthracis, which it somewhat resembles morphologically, and also the lesions produced by it. He found that it grew only in anaerobic conditions, but was able to cultivate it only in an impure state. A similar organism was later more fully studied by Koch, which he considered to be the same as Pasteur's *vibrion septique*. He pointed out, however, that the disease produced by it is not really a septicæmia, as immediately after death the blood may be free from the bacilli. Accordingly he gave to it the name "bacillus of malignant œdema." The two names are now used as synonymous, though it is not possible to say whether the organisms originally described by Pasteur and by Koch were identical.

In pre-war times "malignant œdema" in the human subject

was usually described as a spreading inflammatory œdema attended with emphysema, and ultimately followed by a certain amount of gangrene. In only some cases of this nature, however, is the bacillus of malignant œdema present, and it is usually associated with other organisms which aid its spread. One of us, however, observed a fatal case in which the bacillus was present in pure condition. Here there occurred intense œdema with swelling and induration of the tissues, and the formation of vesicles on the skin. These changes were attended with a reddish discoloration, afterwards becoming livid. Emphysema was not recognisable until the very tense limb was incised, when it was detected, though in small degree. Further, the tissues had a peculiar heavy, but not putrid, odour. The bacillus, which was obtained in pure culture, was present in enormous numbers in the affected tissues, attended by cellular necrosis and serous exudation. The picture, in short, corresponded with that seen on inoculating a guinea-pig with a pure culture.

During the war the organism has been found in putrid wounds and cases of gas gangrene. M'Intosh, in fact, found it to be next in order of frequency to the B. welchii in gangrenous wounds. Weinberg places it along with the latter organism as a cause of "classical gas gangrene," though it is much less common, and usually occurs in association with other organisms. He, moreover, states that cases of pure infection are rare, and that in these emphysema is not a striking feature, gas occurring only in the deeper tissues in small bubbles, and sometimes only recognisable at operation. These features accordingly correspond with those in the case referred to.

Microscopical Characters.—The *vibrion septique*, or B. œdematis maligni (*Clostridium œdematis maligni*), is a comparatively large organism, being slightly less than 1 μ in thickness, that is, thinner than the anthrax bacillus. It usually occurs in the form of single rods 3 to 10 μ in length, but both in the tissues and in cultures in fluids it frequently grows out into long filaments, which may be uniform throughout or segmented at irregular intervals. In cultures on solid media it chiefly occurs in the form of shorter rods with somewhat rounded ends. The rods are motile, possessing several laterally placed flagella. Motility is usually well marked in the serous exudate of the lesions, but in cultures only a few bacilli may show active movement. Under suitable conditions they form spores, which have an oval shape, their thickness somewhat exceeding that of the bacillus; they are central or subterminal in position. In acute

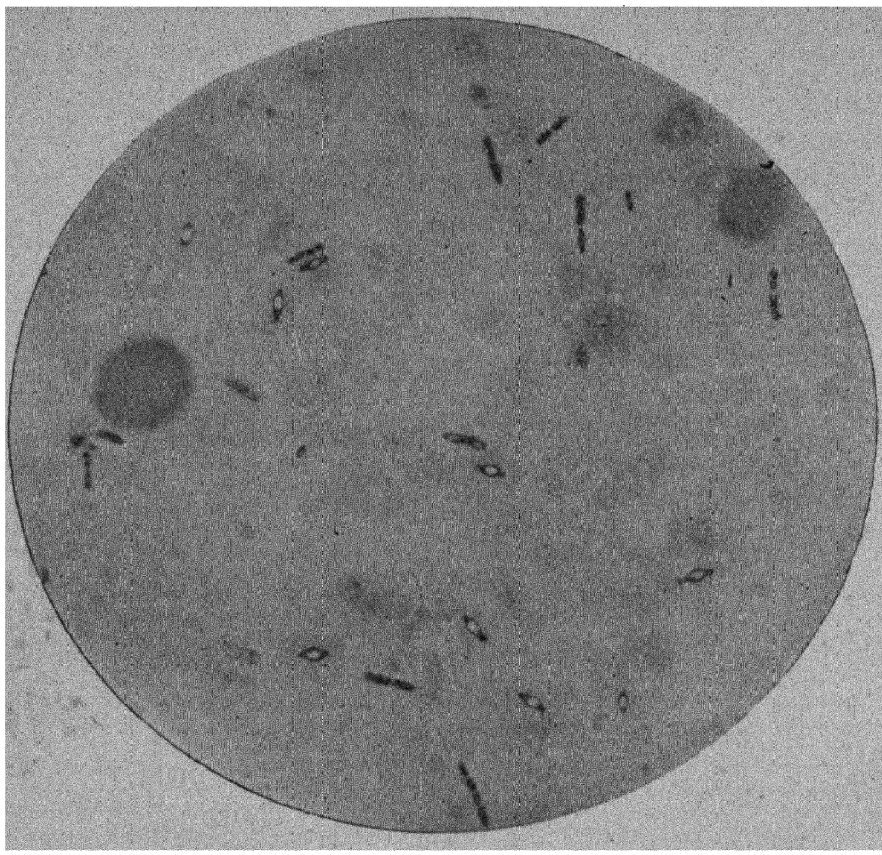


FIG. 151.—Film preparation from the affected tissues in a case of malignant œdema in the human subject, showing the spore-bearing bacilli. Gentian-violet. ×1000.

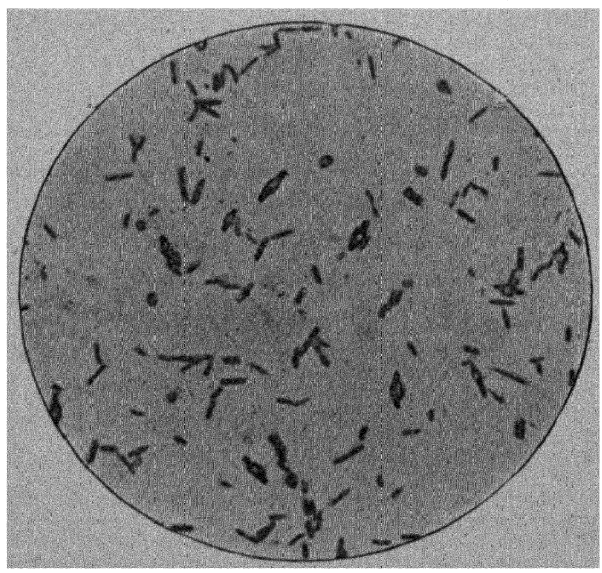


FIG. 152.—Bacillus of malignant œdema, showing spores. From a culture in glucose agar, incubated for three days at 37° C. Stained with weak carbol-fuchsin. ×1000

spreading lesions the bacilli are usually free from spores, but at a later period they may be found (Fig. 151). The bacillus can be readily stained by any of the basic aniline stains. It is Gram-positive, but in older cultures Gram-negative forms occur.

Characters of Cultures.—This organism is a strict anaerobe; it grows readily at the room temperature, but the optimum is the temperature of the body.

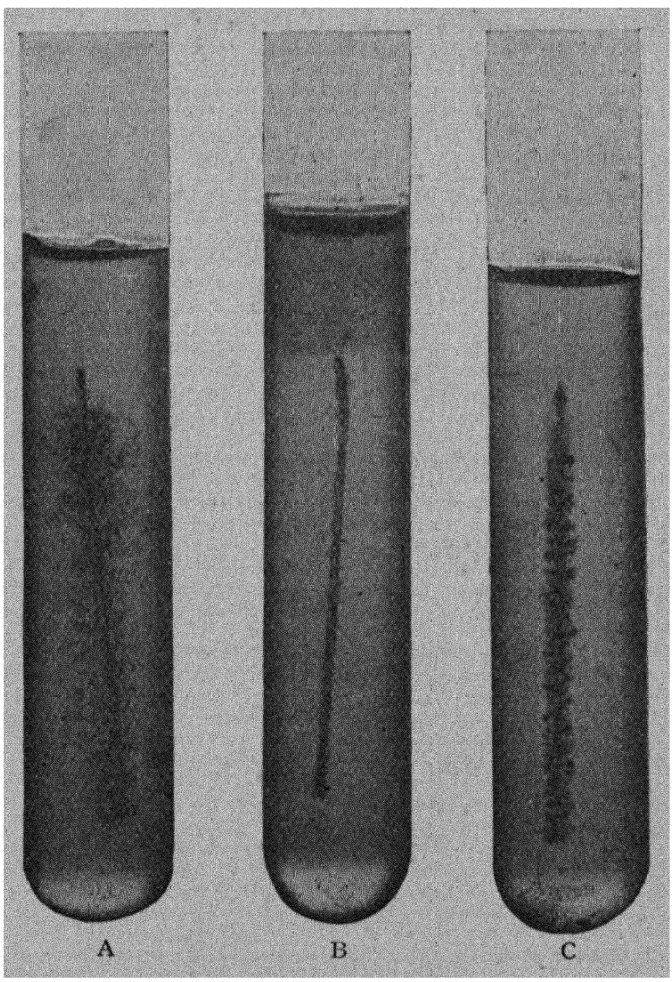


FIG. 153.—Stab cultures in agar, five days' growth at 37° C. Natural size.

A. Tetanus bacillus. B. Bacillus of malignant œdema. C. Bacillus of quarter-evil (Rauschbrand).

In deep tubes of glucose agar at 37° C. growth is extremely rapid. Along the line of puncture, growth appears as a somewhat broad whitish line, with short lateral projections here and there (Fig. 153, B). Gas may be formed, but this is most marked in a shake culture. The individual deep colonies are woolly in appearance without definite centre, whilst the super-

ficial ones are thin discs with irregular peripheral radiations. The growths generally are like those of the B. tetani, but have a somewhat coarser character. Cultures in gelatin present somewhat similar features, and the deep colonies have been compared to those of the B. subtilis ; liquefaction of the medium follows. The cultures possess a peculiar heavy, though not putrid, odour. M'Intosh found that the organism ferments glucose, maltose, and lactose, but not saccharose, inulin, glycerin, or starch. His strains produced coagulation of milk, but without any digestion of the casein, and caused no liquefaction of coagulated serum ; in cooked meat medium there was no change in colour. He accordingly places the organism in the non-proteolytic group. Spore formation occurs in cultures above 20° C., and is usually well seen within forty-eight hours at 37° C.

Experimental Inoculation.—A considerable number of animals—the guinea-pig, rabbit, dog, sheep, and goat, for example—are susceptible to inoculation with this organism. There is general agreement as to its marked pathogenic properties. Especially is this the case when the serous exudate containing the bacillus is used for inoculation, a mere fraction of a cubic centimetre being a fatal dose. M'Intosh found that with his strains 0·01 c.c. of a fluid culture injected intramuscularly killed a guinea-pig within twenty-four hours.

Subcutaneous inoculation with pure cultures produces in the guinea-pig chiefly a widespread gelatinous œdema, and a blood-stained serous fluid exudes from the affected part. The underlying muscles are softened and partly necrosed, and of bright red colour ; but there is little formation of gas, and putrid odour is almost absent. The internal organs show little change. The bacilli are present in the peritoneal fluid, and occur as long motile filaments. They have sometimes been cultivated from the blood, but they are always scanty. Infection with the organism is said to occur frequently when a little garden-earth is introduced subcutaneously in the guinea-pig, but in this case the local lesion presents a putrid character, owing to the presence of other organisms.

When the bacilli are injected into mice, however, they enter and multiply in the blood stream, and they are found in considerable numbers in the various organs, so that a condition not unlike that of anthrax is found. The spleen also is much swollen.

Immunity.—Malignant œdema was one of the first diseases against which immunity was produced by injections of toxins. The filtered cultures of the bacillus in sufficient doses produce death with the same symptoms as those caused by the living

organisms, but a relatively large quantity is necessary. Roux and Chamberland (1887) found that if guinea-pigs were injected repeatedly with non-fatal doses of cultures sterilised by heat or freed from the bacilli by filtration, immunity against the living organism could be developed in a comparatively short time. They found that the filtered serous exudate of animals dead of the disease is more highly toxic, and also gives immunity when injected in small doses. These experiments were confirmed by Sanfelice.

Bacillus œdematiens (*Clostridium œdematiens*).—This organism, first described by Weinberg and Ségum, has the following characters. It is a fairly large bacillus, of about the same size as the B. welchii, but tending to be rather longer. It is distinctly pleomorphous, often occurring in C and S forms, and growing also in chains. It is Gram-positive, but Gram-negative forms are found in older cultures. It possesses numerous lateral flagella, though in ordinary conditions motility is practically absent. Spore formation occurs, the spores being usually subterminal in position.

Cultivation.—The organism grows well on all the ordinary media down to a temperature of about 20° C., but only under strict anaerobic conditions. On solid media the deep colonies are small, somewhat irregular balls with woolly margin, while the superficial ones are film-like with wavy border. In milk it causes the formation of a loose acid clot, which falls to the bottom as a grumous deposit; there is no digestion of the casein, nor is there any liquefaction of gelatin or digestion of coagulated serum. It actively ferments nearly all the ordinarily used sugars with evolution of gas. In cooked meat medium it produces a pink colour, which afterwards fades almost to a white, and there is slight formation of gas. The bacillus may thus be regarded as belonging to the saccharolytic type of anaerobes.

Pathogenic Effects.—In a series of cases of gas gangrene Weinberg found the B. œdematiens to occur next in order of frequency to the B. welchii, and he considers it to be the most important agent in what he calls the "toxic form" of gas gangrene. This type is characterised by a rapidly spreading gelatinous œdema, with little or no gas formation, and by severe symptoms of general poisoning. Blood culture usually gives a negative result, though the bacillus may be found in the blood after death. Intramuscular or subcutaneous injection in the guinea-pig gives a similar picture, the chief feature being the extent and thickness of the œdema; the lesion has no putrid odour. The organism was shown by Weinberg to form a soluble toxin, which in the case of some strains is very potent. Injection of a filtrate from a fluid culture reproduced the characteristic œdema in the guinea-pig. He also produced an antitoxin which was efficient when tested experimentally, and which was used in some cases of the human infection, apparently with success.

Bacillus tertius (*Clostridium tertium*).—This is another saccharolytic bacillus, but with *terminal spores*. It is common in contaminated wounds, and the name was given by Henry, as he found

it to be third in order of frequency among the anaerobes. It is regarded as being probably the same as the bacillus IX of von Hibler and the bacillus Y of Fleming. The B. tertius is a fairly long and thin bacillus, and is often somewhat curved; it is Gram-positive, but the power of retaining the stain is soon lost in cultures. It is feebly motile or non-motile. The spores are terminal; the small forms are round, and stain deeply with a basic dye; the larger are oval, racquet-shaped, sometimes of considerable length. Occasionally a spore is present at each end of a bacillus. The superficial colonies are round, semi-transparent discs, which do not become large; the deep colonies are of lenticular shape; occasionally, from both, small offshoots occur. On a moist surface there is a tendency for the growth to spread as a thin film. In milk a small amount of gas is produced, and a day or two later a soft friable coagulum. In cooked meat medium both acid and gas are formed; later the fluid becomes clear and the meat assumes a pink colour. There is no liquefaction of gelatin or coagulated serum. The organism has wide fermentative action when tested on various carbohydrates, but different strains vary in this respect. It has practically no pathogenic effects when tested experimentally, though it probably gives rise to gas-formation in wounds.

The following organisms are examples of the proteolytic group:

Bacillus sporogenes (*Clostridium sporogene*).—This organism, which was first separated from fæces by Metchnikoff and described by him, is probably the commonest anaerobe in cultivated soil. It is present in the great majority of putrid wounds, and, owing to its rapid growth and spore formation, often interferes with the separation of other anaerobes. It is a fairly large bacillus, of about the same length as the B. welchii, but thinner, and usually occurs as single elements. It is Gram-positive, but, as is common with members of the group, Gram-negative forms are to be found in older cultures. Spore-bearing forms are common in wounds, and in cultures spores are formed with great rapidity, so that they may be seen within twenty-four hours (Fig. 154). The spores, which have very high powers of resistance, are usually subterminal, though occasionally central in position. The organism possesses

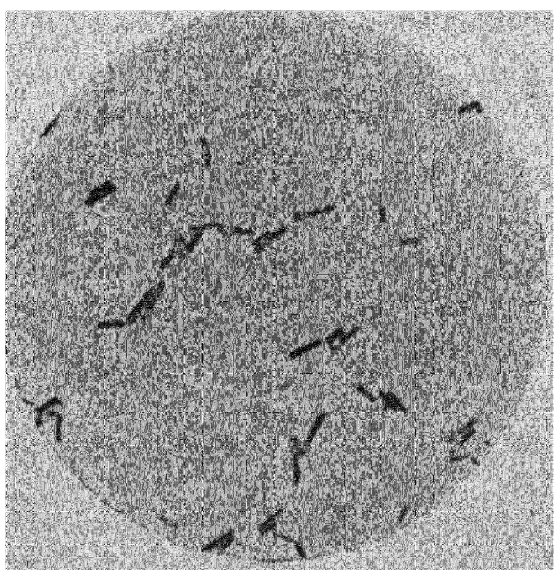


FIG. 154.—B. sporogenes, pure culture, showing subterminal spores. Stained with carbol-thionin blue. ×1000.

numerous lateral flagella, and most strains are actively motile. It grows readily under anaerobic conditions, and the cultures have a markedly putrid odour. In deep glucose agar tubes the growth forms a thick line, from which there are short and stout lateral offshoots, attended by abundant gas formation, while individual colonies are small balls with woolly margin. Superficial colonies have a granular centre and present an arborescent appearance at the edge. The organism rapidly liquefies gelatin and coagulated serum, and also pieces of coagulated white of egg. In cooked meat medium there is evolution of gas and rapid digestion; the meat assumes a dirty, purplish tint, and ultimately becomes blackened. In milk there is a precipitation of casein without actual coagulation, and then digestion rapidly follows. The organism ferments glucose, lævulose, and maltose, but none of the other sugars ordinarily used. The organism is thus seen to have marked proteolytic properties, and it has been shown to form amino-acids, and as final products ammonia, sulphuretted hydrogen, and various volatile substances. It forms large quantities of butyric acid even in sugar-free media (Wolf and Harris).

The B. sporogenes has little or no pathogenic properties when injected in animals; and observations on gun-shot wounds supply no evidence that it invades the healthy tissues. It may be regarded chiefly as a proteolytic saprophyte which grows on dead and dying tissues and brings about digestive softening and putrefactive changes. The B. sporogenes is closely allied to another proteolytic and putrefactive anaerobe described by Bienstock under the name B. putrificus.

Bacillus histolyticus (*Clostridium histolyticum*).—This is another proteolytic and putrefactive anaerobe separated by Weinberg from cases of gas gangrene. It is 2–6 μ in length and rather thinner than the B. welchii; it is often arranged in pairs. It is Gram-positive and forms large oval subterminal spores. The surface growth is in the form of a very thin film, with offshoots at the margin. Its action on milk and coagulated serum is similar to that of the B. sporogenes, but is even more rapid. In cooked meat medium also it produces very rapid digestion, with foul odour, and one feature described by Henry is the separation of white balls of acicular crystals which are probably tyrosin—an appearance which is apparently characteristic of this organism. The cultures have a foul odour. A striking evidence of the proteolytic action of this organism is seen when it is injected subcutaneously in a guinea-pig. A rapid digestion of the tissues in the vicinity occurs, so as sometimes actually to expose the bones.

Quarter-evil (German, Rauschbrand; French, Charbon Symptomatique)

The characters of the bacillus (*Clostridium chauvœi*) need be only briefly described, as, so far as is known, it never infects the human subject. The natural disease, which occurs especially in certain localities, affects chiefly sheep, cattle, and goats. Infection takes place by some wound of the surface, and then spreads in the region around, attended by inflammatory swelling, bloody œdema, and emphysema of the tissues. The part becomes greatly swollen,

and of a dark, almost black, colour. Hence the name "black-quarter" by which the disease is often known. The bacillus which produces this condition is present in large numbers in the affected tissues, associated with other organisms, and also occurs in small numbers in the blood of internal organs.

The bacillus morphologically closely resembles that of malignant œdema. Like the latter, also, it is a strict anaerobe, and its conditions of growth as regards temperature are also similar. It is, however, somewhat thicker, and does not usually form long filaments; occasionally it occurs in short chains. The spores, which are of oval shape and broader than the bacillus, are usually subterminal, though central-spored clostridium forms occur (Fig. 155). This bacillus is actively motile, and possesses numerous lateral flagella. The characters of the cultures, also, resemble those of the bacillus of malignant œdema, but in a stab culture in glucose agar there are more numerous and longer lateral offshoots, the growth being also more luxuriant (Fig. 153, C.). The superficial colonies are small greyish rounded discs with a thicker centre; the deep colonies show a radiating appearance at the periphery. M'Intosh found that the organism belongs to the non-proteolytic class. It produces acid and clot in milk in three to four days and ferments glucose, maltose, lactose, and saccharose, but not inulin or dulcite. It does not liquefy coagulated serum.

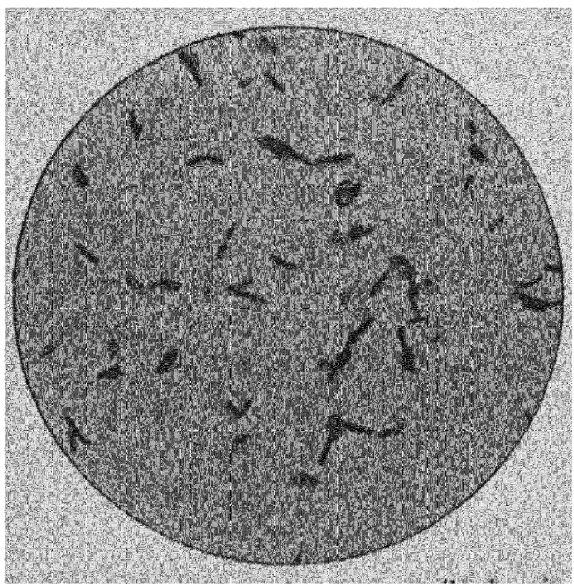


FIG. 155.—Bacillus of quarter-evil, showing spores. From a culture in glucose agar, incubated for three days at 37° C. Stained with weak carbol-fuchsin. ×1000.

The disease can be readily produced in various animals, *e.g.* guinea-pigs, by inoculation with the affected tissues of diseased animals, and also by means of pure cultures, though an intramuscular injection of a considerable amount of the latter is sometimes necessary. The condition produced in this way closely resembles that in malignant œdema, though there is said to be more formation of gas in the tissues. Rabbits are more resistant to this disease, whilst they are comparatively susceptible to malignant œdema. As in the case of tetanus, inoculation with living spores which have been deprived of adherent toxin by heat does not produce the disease. A toxin can be separated by filtration from cultures of bouillon containing 5 per cent. glucose and a thick emulsion of sterile calcium carbonate. It is fairly resistant to heat, withstanding two hours at 70°–75° C. without being destroyed, and it is also very rapid in its action, being capable in appropriate dose of killing a horse in five minutes. It is to be noted as an important fact, that while freshly isolated cultures possess a high

degree of virulence they may have little capacity for toxin production *in vitro*. Grassberger and Schattenfroh state that there may be an antagonism between maximum virulence and maximum toxin production. One of the properties of the toxin is said to be a capacity for killing leucocytes.

The disease is one against which immunity can be produced in various ways, and methods of preventive inoculation have been adopted in the case of animals liable to suffer from it. This subject was specially worked out by Arloing, Cornevin, and Thomas, and later by others. Immunity may be produced by injection (especially by the intravenous and intraperitoneal routes) of a non-fatal dose of the virus (*i.e.* the œdematous fluid found in the tissues of affected animals, which contains the bacilli), or by injection with larger quantities of the virus attenuated by heat, drying, etc. It can be produced also by cultures attenuated by heat and by the products of the bacilli obtained by filtration of cultures. An antitoxin has been produced against the toxins of the bacillus, and a method of protection in which the action of this antitoxin is combined with that of the virus has been used (cf. Anthrax, p. 393). The antitoxin is said to increase the chemotactic properties of the leucocytes.

Fusiform Anaerobic Bacilli Pathogenic to Man

Babés in 1884 described organisms of this type in a diphtheria-like affection of the fauces, and since that time the presence of similar organisms has been noted in necrotic inflammations, ulcerative stomatitis, noma, and like affections. They have also been found in pulmonary lesions and in abscesses in other parts of the body ; in these the pus is very foul-smelling. The association of fusiform bacilli with a form of angina has been specially recognised since the work of Vincent (1898–99) ; and this condition often goes now under the name of " Vincent's angina." He recognised two forms of the affection—(*a*) a diphtheroid type, characterised by the formation of a firm yellowish-white false membrane, very like that of diphtheria, associated with only superficial ulceration ; and (*b*) an ulcerative type, where the membrane is soft, greyish, and foul-smelling, attended with ulceration and surrounding œdema. In the former type fusiform bacilli are present alone ; in the latter, which is distinctly the commoner, there are also spirochætes. The fusiform bacilli are thin rods measuring on the average 10 to 14 μ in length, and less than 1 μ in thickness ; they are straight or slightly curved, and are tapered at their extremities. The central portion often stains less deeply than the extremities, and not infrequently shows unstained points and granules (Fig. 156 ; Plate I., Fig. 4). The organisms are non-motile. They stain fairly deeply with Löffler's methylene-blue or with

weak carbol-fuchsin, and are Gram-negative. The spirochætes are long delicate organisms showing several irregular curves, and are motile; in appearance they resemble the treponema refringens and similar organisms found in gangrenous conditions. They stain less deeply than the bacilli. Sometimes they are numerous, sometimes scanty. In a section through the false membrane, when stained with methylene- or thionin-blue, there is usually to be seen a darkly stained band, a short distance below the surface, which is due to the presence of large

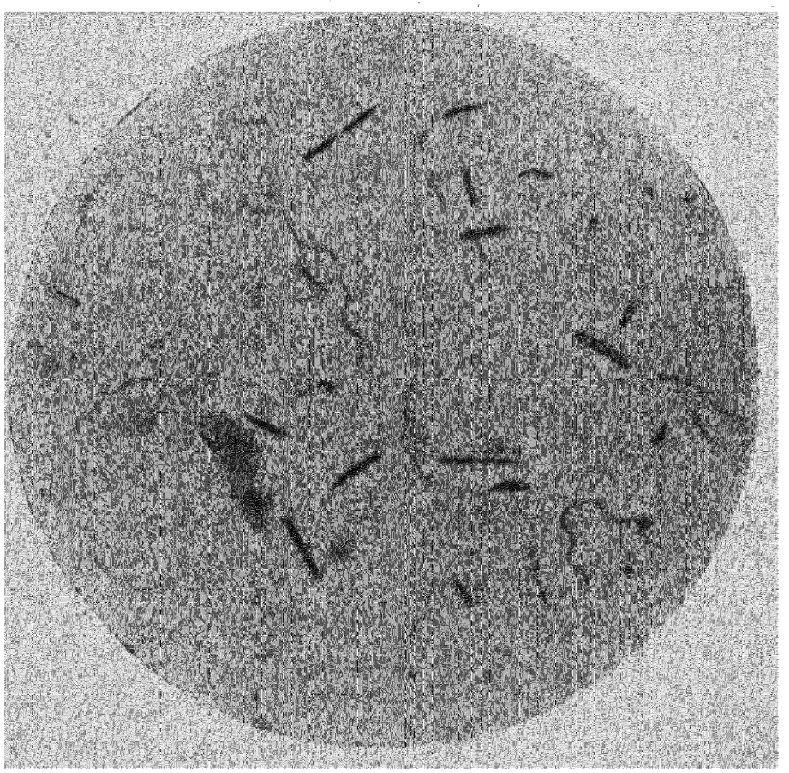


FIG. 156.—Film preparation from a case of Vincent's angina, showing fusiform bacilli and spirochætes. Stained with weak carbol-fuchsin. ×1000.

masses of the fusiform bacilli closely packed together; neither they nor the spirochætes appear to pass deeply into the tissues. It is also to be noted that fusiform bacilli are sometimes present in the secretions of the mouth in normal conditions, and may occur in increased numbers in true diphtheria. Vincent's results have been confirmed by others, and there is no doubt that fusiform bacilli, of which there are probably several species, are associated with various spreading necrotic lesions. During the war, cases of Vincent's angina were of common occurrence and were met with in small epidemics. Ulcerative gingivitis and

stomatitis have been found to be associated with the presence of the same organisms, and in some cases these lesions precede the infection of the fauces. It would be advisable to apply the term " Vincent's disease," as suggested by Bowman, so as to include all the lesions produced by the organisms in question. In phagedænic lesions of the genitals, fusiform bacilli are usually present, with or without spirochætes, though in our experience they are as a rule of smaller size than those met with in the throat. Cultures of fusiform bacilli have been obtained by Ellermann, by Weaver and Tunnicliff, and by others. They grow only under anaerobic conditions, and the best media are those consisting of a mixture of serum or blood and agar (1 : 3). The organisms form small rounded colonies of whitish or yellowish colour, somewhat like those of a streptococcus, but rather felted in appearance on the surface. In cultures, especially in fluid media, undulating filamentous forms may be found, but there does not appear to be sufficient evidence that the fusiform bacilli and the spirochætes found in angina are stages in the development of the same organism, as was maintained by Tunnicliff. Krumwiede and Pratt, for instance, found that the filamentous forms were not true spirochætes but merely represented variations in morphology. These observers studied the fermentative properties of the B. fusiformis and found that it fermented various sugars without the formation of gas ; they distinguished two strains, one fermenting saccharose, the other not. Injections of pure cultures in animals sometimes produce suppuration but never necrosis (Ellermann).

XXII

DISEASES DUE TO SPIROCHÆTES: SYPHILIS AND YAWS

Introductory.—The general morphology of the spirochætes and their resemblance in certain particulars to protozoa have already been discussed (pp. 2, 14). In the new classification of the Society of American Bacteriologists they are designated Spirochætales and included as an order of the Schizomycetes, *i.e.* in the plant kingdom. In some instances pathogenic species are transmitted directly, but in others blood-sucking ectoparasites are concerned and it has been suggested that some phase of a supposed life cycle may occur in the intermediate insect host.

The parasitic members of the order may be classified into the two main genera, Treponema and Leptospira (Dobell). The species of the former, which show undulating motion, vary in their curving; in some cases the organism presents the appearance of a perfect and regular spiral, *e.g.* the *Treponema pallidum* of syphilis. To this type the term "Treponema" has been restricted by the Society of American Bacteriologists. In other species, *e.g.* the organisms of the relapsing fevers, the coils or spirals are less regular, and instead of a definite spirality the organisms appear as filaments with curves or undulations. Organisms of this second type have been placed in a separate genus, *Borrelia* (formerly *Spironema*) by the Society of American Bacteriologists. The organisms of the genus Leptospira exhibit a very delicate spiral structure with numerous fine coils which are best seen by dark-ground illumination and may not be so apparent in stained preparations. The organisms possess characteristic hooked ends, and in moving, the ends show a rotatory action, while the rest of the organism tends to remain rigid.

The human diseases produced by spirochætes—the *Spirochætoses*—can be classified into certain main groups: (1) syphilis and yaws, (2) the spirochætal relapsing fevers, (3) certain

ulcerative and gangrenous conditions with which spirochætes are associated, usually along with a characteristic fusiform bacillus (B. fusiformis), *e.g.* Vincent's angina, gangrenous balanitis, etc., (4) infectious jaundice (Weil's disease), and yellow fever, due to leptospira types, (5) rat-bite fever, due to a spiral organism which appears to be more related to the spirilla, though it has usually been classified with the spirochætes. Certain of these diseases are essentially blood infections, *e.g.* the relapsing fevers, and the spirochæte is transmitted by blood-sucking insects. Others are due to organisms which are primarily tissue parasites, *e.g.* the Treponema pallidum—blood invasion, when it occurs, being a later phenomenon—and infection occurs by direct contact. Infectious jaundice seems to occupy a somewhat intermediate position, as the organisms occur in the blood stream, but tend to settle and flourish in certain organs.

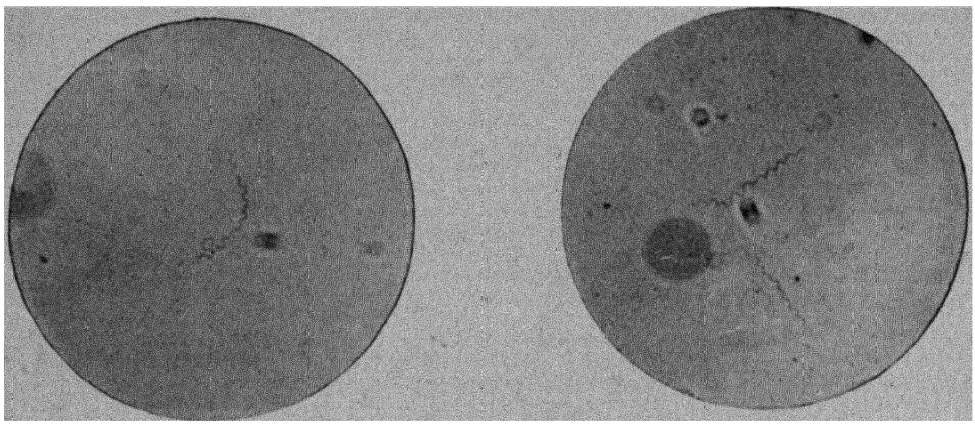
It should also be noted that certain types of spirochætes are common commensal organisms and some of them may simulate pathogenic types. These will be referred to in relation to the pathogenic types which they resemble.

SYPHILIS

The cause of syphilis is the organism discovered by Schaudinn and Hoffmann in 1905 and called by them the *Spirochæta pallida*, now known as the *Treponema pallidum*, though the term *Spironema pallidum* is also used. They described its characters and its occurrence in syphilitic lesions, and their observations have been fully confirmed. Its recognition, at first somewhat difficult, has been rendered comparatively easy by the introduction of new methods.

Treponema pallidum (*Treponema pallidum, Schaudinn and Hoffmann*).—This is a minute spiral-shaped organism, showing usually from six to fourteen curves, though longer forms are met with ; the curves are small (each measuring a little over 1 μ), comparatively sharp, and regular (Figs. 157, 158, 159). It may be said to measure 4 to 14 μ in length, while it is extremely thin, its thickness being only 0·25 μ. In a fresh specimen, say, in exudate from a chancre, the organism shows active movements, which are of three kinds—rotation about the long axis, gliding movements to and fro, and movements of flexion of the whole body ; there is little actual locomotion, and a specimen will often remain in the same field for a long time. The ends

are pointed and tapered to a flagellum-like structure. Both in fresh specimens and in dried films (Figs. 157–159) the regularity



FIGS. 157 and 158.—Film preparations from juice of hard chancre showing Treponema pallidum. Giemsa's stain. ×1000. (From preparations by Dr. A. MacLennan.)

of the spirals is well maintained, though in the latter there is sometimes distortion or drawing out of a spiral. The use of

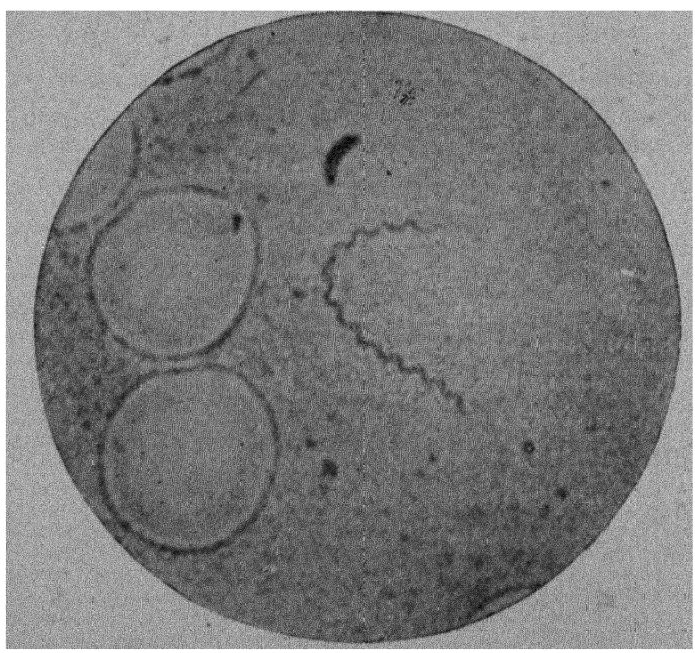


FIG. 159.—Film preparation from juice of hard chancre showing Treponema pallidum. Giemsa's stain. ×2000. (From a preparation by Prof. Haswell Wilson.)

dark-ground illumination (p. 97) is of great service in searching for the organism.

In ulcerated syphilitic lesions, and also in non-syphilitic

lesions of the genitals, other organisms are, of course, present, and not infrequently various other spirochætes; different varieties of the latter may also be met with normally on certain parts of the body surface, *e.g.* in the mouth and on the genitalia in both sexes, also in the fæces. Of these, several species have been described, e.g. *Tr. refringens, Tr. gracile, Tr. minutum.* Tr. refringens is a comparatively coarse organism, more highly refractile, while its curves vary during the movements; in film preparations the curves appear irregular or are lost to a large extent (Fig. 163). It is similar in appearance to the spirochætes



FIG. 160.—Section of spleen from a case of congenital syphilis, showing several examples of Treponema pallidum. Levaditi's method. ×2000.

of Vincent's angina (p. 560). It is found on ulcers on various parts of the body, and may occur in the sputum in cases of bronchitis. Some of the other species resemble Tr. pallidum more closely in the regularity of their curves, *e.g.* Tr. gracile, but are larger or differ in the character of their movements. Tr. minutum, isolated by Noguchi from the secretions of the external genitalia, resembles Tr. pallidum closely. We believe that in the case of genital lesions there is little difficulty to the experienced observer in recognising the Tr. pallidum, provided that the superficial organisms are removed and the lymph is taken from the lesion for examination. These organisms generally stain deeply with Giemsa's stain and are of a bluish tint:

the Tr. pallidum is coloured a faint pink. In lesions of the mouth, and probably in some others, *e.g.* fœtid ulcerations, etc., and also normally in the mouth (between the teeth) there occur, however, spirochætes which are indistinguishable morphologically from the Tr. pallidum, *e.g.* the Tr. microdentium (Fig. 161) and Tr. mucosum; these may be specially abundant in carious teeth and pyorrhœa alveolaris. Both of these organisms have been cultivated by Noguchi; they have been proved to be devoid of pathogenic properties, and the cultures, moreover, have a foul odour. The Tr. pertenue of yaws (p. 574) has also the same microscopical appearances. In the microscopical diagnosis of the organism of syphilis, just as in the case of the tubercle bacillus (p. 332), an all-important point is, accordingly, the source of the organism; and we may say that if we except the case of yaws, which does not occur in this country, an organism with the characters described above can be identified with certainty as the Tr. pallidum provided that it is obtained from the *substance* of the tissue lesion.

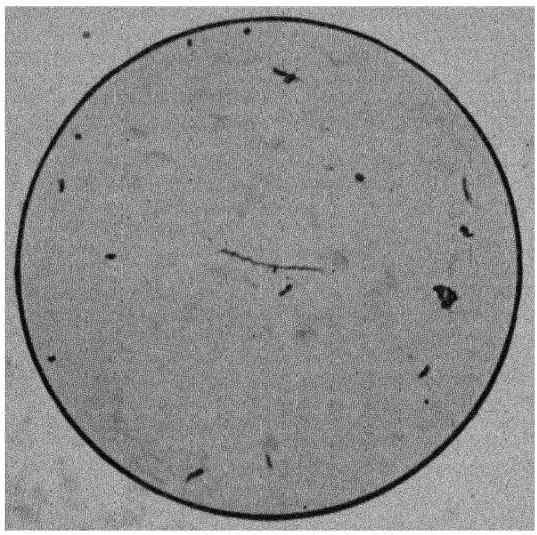


FIG. 161.—*Tr. microdentium.* Film preparation from mouth showing characteristic morphology of the organism. Stained by Becker's method. ×1000.

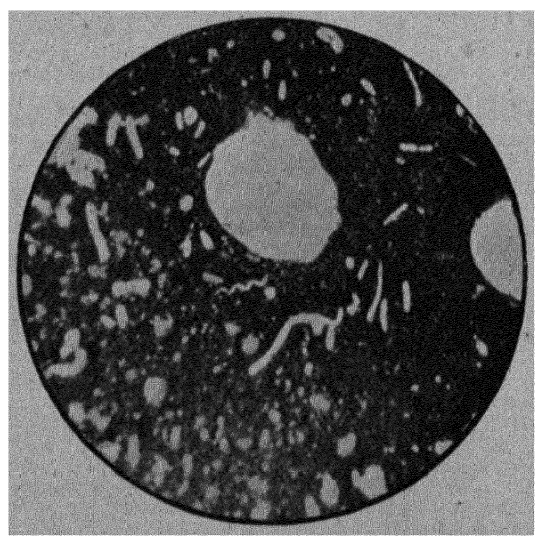


FIG. 162.—*Tr. gracile* (*Tr. calligyrum*). Film preparation from balanitis pus treated by Indian ink method. The organism, with regular spirals, is seen in the centre of the field. ×1000.

Noguchi, on studying different strains of the Tr. pallidum in cultures, found that they varied in thickness, and he was able to

distinguish thick, thin, and intermediate types. He also found that they differed in their pathogenic action, the thick forms on injection into the testicle of a rabbit causing nodular lesions of cartilaginous hardness, the thin forms producing a diffuse indurative lesion. Levaditi and Marie also hold that there are " dermotropic " and " neurotropic " strains of Tr. pallidum, which differ in their pathogenic properties. These observations are suggestive as possibly throwing some light on the variations in the effects in the human subject; but they have not been confirmed by others.

As regards the distribution of the Tr. pallidum, the following is a summary of the results of many investigations. In the primary sore in the stage of active ulceration and in the related lymphatic glands, the juice of which can be conveniently obtained by means of a hypodermic syringe, the organism has been found in a very large majority of cases. It has been also obtained in the papular and roseolar eruptions, in condylomata and mucous patches—in fact, one may say generally, in all the primary and secondary lesions. Schaudinn in his last series of cases, numbering over seventy, found it in all, and on a few occasions detected it in the blood during life in secondary syphilis. It has also been obtained from the spleen during life. In the congenital form of the disease the organism may be present in large numbers (Plate II., Fig. 6), as was first shown by Buschke and Fischer, and by Levaditi. In the pemphigoid bullæ, in the blood, in the internal organs, the liver, suprarenals, lungs, spleen, and even in the heart its detection may be comparatively easy, owing to the large numbers present (Fig. 160). In the organs in congenital syphilis the spirochætes are chiefly extra-vascular in position, but many may occur in the interior of the more highly specialised cells, for example, liver cells. They also abound sometimes on mucous surfaces, *e.g.*, of the bladder and intestine in cases of congenital syphilis. The enormous numbers of the organism which may be present in a well-preserved condition in macerated fœtuses render it probable that the organism may multiply in the dead tissues under anaerobic conditions. It is also present in syphilitic placentæ, though not usually in large numbers. It has been

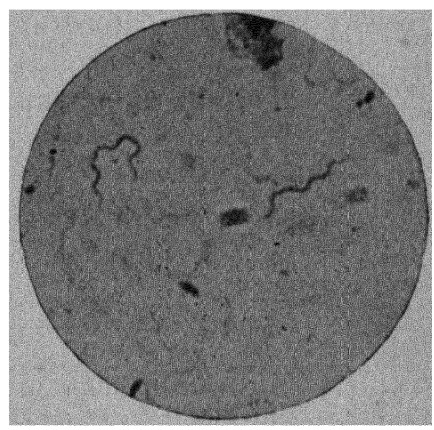


FIG. 163.—Treponema refringens in film preparation from a case of balanitis. ×1000.

generally supposed that tertiary syphilitic lesions are non-infective, and the results of the earlier observations on the Tr. pallidum were apparently in accordance with this view, as they gave negative results. More prolonged search has, however, shown that the organism may occur in tertiary lesions also. It has been found to be present in the peripheral parts of gummata, especially at an early stage of their formation ; and the observations of Schmorl, Benda, J. H. Wright, and others show that it is often to be found in syphilitic disease of arteries, sometimes occurring in considerable numbers in the thickened patches in the aorta. That the spirochætc may persist in the body for a very long time after infection, has been abundantly shown by different observers ; in one case, for example, its presence was demonstrated sixteen years after the primary lesion. It can readily be demonstrated in sections of syphilitic lesions by the method described on page 114. Noguchi and Moore discovered the spirochæte in the brain in general paralysis of the insane in a certain proportion of cases. The organism was seen in all the layers of the cerebral cortex, with the exception of the outermost, and the cases in which it was found had run a relatively rapid course. Infection has also been transmitted to the rabbit (*vide infra*) by inoculation with the brain tissue of general paralytics.

Cultivation.—Although Muhlens and Hoffmann had previously obtained pure cultures of an organism morphologically identical with the Treponema pallidum, it is chiefly to Noguchi that we owe the methods of cultivation. We shall accordingly state his results, which in certain respects differ from those of the other two observers. In the first instance his cultures were made from syphilitic lesions in the rabbit, but later directly from the lesions of the human disease. As a culture medium he used a mixture of 2 parts of 2 per cent. agar and 1 part of ascitic or hydrocele fluid, to which a small portion of sterile rabbit's kidney or other organ was added, the medium being placed in deep tubes and covered with a thick layer of paraffin oil or vaselin. The medium was inoculated through the oil, the maintenance of strict anaerobiosis being essential. The contaminating bacteria which were present formed a thick growth along the line of inoculation, whilst the spirochætes grew as a diffuse haze into the surrounding medium. By making subcultures from parts apparently free from bacterial growth he succeeded in obtaining the organism in the pure condition. At first the organisms were small, but after several days they had the usual length of the Tr. pallidum and all its characteristics. He stated that the organism multiplies by longitudinal division, but this is not generally accepted. Pure cultures may be grown in suitable (unheated) ascitic fluid, sufficient being placed in an $8 \times \frac{1}{2}$ inch test-tube to half fill it, to which is added a piece of fresh sterile rabbit's kidney the whole then being covered by a

half-inch layer of sterile vaselin (*Smith-Noguchi medium*). On inoculating monkeys (*Macacus* and *Cercopithecus*) by scarification, in some cases indurated syphilitic papules developed and the blood of the animals gave a positive Wassermann reaction. The etiological relation of the organism has thus been completely established. But the cultivation of Tr. pallidum presents great difficulties, and the cultures obtained have usually been without pathogenic action.

Transmission of the Disease to Animals.—Although various experiments had previously been made from time to time by different observers, in some cases with reported successful result, it is to the work of Metchnikoff and Roux (1903–05) that we owe most of our knowledge. These observers carried on a large series of observations, and showed that the disease can be transmitted to various species of monkey. Of these the anthropoid apes are most susceptible, the chimpanzee being the most suitable for experimental purposes. Their results have been confirmed by Lassar, Neisser, Kraus, and others. The number of experiments on these animals is now very great, and the general result is that the disease has been transmitted by material from all the kinds of syphilitic lesions in which spirochætes have been demonstrated, including tertiary lesions and the blood in secondary syphilis. Inoculation is usually made by scarification on the eyebrows or genitals; but other parts of the skin are also susceptible in the higher apes. The subcutaneous and other methods of inoculation, with the exception of intratesticular and intravenous, give negative results. The primary lesion is in the form of an indurated papule or of papules, in every respect resembling the human lesion. Along with this there are marked enlargement and induration of the corresponding lymphatic glands. The primary lesion appears on an average about thirty days after inoculation, and secondary symptoms develop in rather more than half of the cases after a further period of rather longer duration. These are of the nature of squamous papules on the skin, mucous patches in the mouth, and sometimes palmar psoriasis. As a rule, the secondary manifestations are of a somewhat mild degree, and in no instance has any tertiary lesion been observed, though this may be due to the animals not having lived long enough. By re-inoculation from the lesions, the disease may be transferred to other animals. The disease may also be produced in macaques and other lower monkeys, but these animals are less susceptible, and, moreover, the inoculation to be successful must be made at the special sites mentioned above; secondary manifestations do not appear. The severity of

the affection amongst apes would in fact appear to be in proportion to the nearness of the relationship of the animal to the human subject. The blood of the infected animals comes to give a positive Wassermann reaction.

As shown first by Hänsell, and afterwards by Bertarelli, the eye of the rabbit is susceptible to inoculation from syphilitic lesions. The material used is introduced in a finely divided state either into the tissue of the cornea or into the anterior chamber, and syphilitic keratitis or iritis, or both, may result, there being a period of incubation of at least two weeks. Levaditi and Yamanouchi have studied the stages in detail, and find that the spirochætes remain in the inoculated material unchanged for a time ; then organisation occurs and the spirochætes multiply, and later still there is a more rapid multiplication and invasion by them of the tissues of the eye. The period of incubation is thus not due to the organism passing through some cycle of development, but simply to its requiring certain conditions for multiplying which are not supplied for some time. The testis of this animal is also a convenient site of inoculation, a syphilitic orchitis being set up, and by this method the disease has been passed through long series of animals. The intratesticular method has proved of great value in testing the infectivity of suspected material, and by this means it has been shown that spirochætes from gummata are not attenuated in virulence. Uhlenhuth and Mulzer produced generalised syphilitic lesions in young rabbits by intra-cardiac inoculation with syphilitic material. They have also found that the organism can pass through the placenta of the rabbit and infect the fœtus.

Tr. cuniculi.—This organism, which is indistinguishable microscopically from Tr. pallidum, is found in naturally occurring chronic ulcers in the neighbourhood of the mouth and genitalia in rabbits. The infection can be transmitted experimentally by scarification and inoculation with material from the ulcers, but only rabbits (not man or other animals) are susceptible. The organisms proliferate abundantly in the deep layers of the epidermis, but they have little tendency to invade the underlying tissues or to spread to the internal organs.

It has long been held that a person suffering from syphilitic disease is not susceptible to a fresh infection, and this has been shown by experimental methods to hold in the artificially produced disease in the ape, the possibility of re-inoculation thus indicating freedom from infection. A considerable number of cases in the human subject have been observed where, after treatment with salvarsan, a second attack of the disease has been

contracted, the inference being that the first attack had been completely cured. In the case of the rabbit, however, it has been found possible to produce a fresh syphilitic lesion when another was still in existence on the cornea. Apparently in this animal the effects of this local lesion do not become general in the same way as in man. On the other hand, intra-testicular inoculation leads to general invasion of the body, and, as Brown and Pearce have shown, metastatic lesions develop. Spontaneous healing tends to occur after the infection has passed through a series of relapses, but even when all signs of the disease have disappeared virulent spirochætes are still present in the body, especially in the lymph glands.

The experimental production of the disease has supplied us with some further facts regarding the nature of the virus. It has been shown repeatedly that the passage of fluid containing the virus through a Berkefeld filter deprives it completely of its infectivity; in other words, it does not belong to the filter-passing group of organisms. The virus is also readily destroyed by heat, a temperature of 51° C. being fatal. On the other hand, defibrinated blood containing Tr. pallidum has been found still to be infective after keeping for forty-eight hours at room temperature. With regard to the production of immunity, very little of a satisfactory nature has so far been established. It has been found that the virus from a macaque monkey produces a less severe disease in the chimpanzee than the virus from the human subject, inasmuch as secondary lesions do not follow; the virus would thus appear to have undergone a certain amount of attenuation in the tissues of that monkey. But accidental infection of the human subject has occurred with spirochætes which had been repeatedly passed through rabbits during a period of over a year and a half. The presence of the spirochæte does not lead to the formation of anti-substances to any marked extent.

Luetin.—Noguchi has prepared an extract from pure cultures of the Treponema pallidum, which he calls *luetin*, and he finds that this gives a characteristic cutaneous reaction in syphilitics. This reaction is analogous to the tuberculin reaction in tuberculosis, and, like it, appears to depend on a condition of supersensitiveness or allergy (p. 229). In a normal individual the intradermic inoculation of luetin produces a local erythema which may sometimes go on to the formation of a slight papule on the second day; thereafter the reaction recedes. In the case of syphilitics Noguchi distinguishes three types of positive reaction—(*a*) *papular form*, in which a large indurated, reddish papule, 5–10 mm. in diameter, forms and increases for three or four days, the colour becoming

dark bluish red; (*b*) *pustular form*, in which the inflammatory change is more severe, the papule changing into a vesicle and then into a pustule; and (*c*) *torpid form*, in which, after a latent period of about ten days, reaction appears and goes on to the formation of a small pustule. Noguchi's claims as to the clinical value of the reaction are supported by other observers. The results obtained so far show that a positive result is got when the disease is latent and often when the Wassermann reaction is negative. It is often absent in secondary syphilis, but may appear after anti-syphilitic treatment has been carried on for some time. Although the reaction may be of value for detecting latent syphilitic infection, it appears that it is not specific in the etiological sense. In positively reacting subjects other substances, *e.g.* agar or killed cultures of bacteria, may produce a similar reaction. Again, non-syphilitic individuals come to react positively as a result of the administration of iodides (Sherrick).

Serum Diagnosis—Wassermann Reaction and Flocculation Reaction.—The method of applying the Wassermann test has already been given (p. 135); we have now to consider the results of its application. It will not be an overestimate to say that a positive result may be obtained in at least 90 per cent. of cases where there is evidence of active general infection. The reaction generally appears first on the tenth to thirtieth day after appearance of the sore, and then gradually becomes more marked; during the period of secondary manifestations it is practically always present; in the tertiary stage with active manifestations a positive result is only a little less frequent. In cases of congenital syphilis with active lesions the proportion of positive reactions is nearly as great as in the secondary stage of the acquired disease. As the disease becomes inactive or is cured the reaction may disappear, but it is to be noted that disappearance of the reaction after being present does not necessarily imply cure of the disease. It may only have become latent, and on its becoming once more active the reaction may reappear; in fact, its presence would appear to be definitely related to the activity of the syphilitic lesions. A positive reaction is practically always present in general paralysis and in the large majority of cases of tabes, and is as frequently given by the cerebro-spinal fluid as by the blood serum in these diseases; in certain cases of neurosyphilis the cerebro-spinal fluid may react positively when the blood serum is negative. As regards other diseases, a positive reaction has been recorded as occurring in leprosy (p. 351) and sleeping-sickness and also in yaws, and occasionally in malaria during the febrile periods; but apart from these diseases it is practically never met with. At present little can be said in explanation of the Wassermann reaction.

It seems to depend on the interaction of lipoidal substances in the extract with proteins in the serum, which are apparently contained in the globulin fraction. It is now accepted that it does not depend on the presence of an immune-body which in association with the causal agent (the spirochæte) fixes complement. Although there is a considerable amount of evidence that the reacting power of the serum is due to an antibody for certain lipoid compounds, we know nothing as to why this should be present in syphilis. The various forms of flocculation reaction (Sachs and Georgi, Meinicke, Dreyer and Ward, Kahn, etc.) give results which are comparable with those of the Wassermann test.

Methods of Examination.—As already said, in the examination of an ulcerated chancre or other lesion it is advisable to get rid of the surface organisms. The surface should be cleansed with saline and dried. A piece of cotton wool soaked in absolute alcohol or spirit is then applied for about a minute; the alcohol is then washed off with saline, and the surface is again dried. After a short time there is usually a free flow of watery lymph, which is practically free from other organisms, and often contains the spirochæte in large numbers; a small drop of this is placed on a slide, a cover-glass is applied, and the specimen is examined by dark-ground illumination. It is advisable to put a thin ring of vaselin on the slide to support the cover-glass. Dried films also may be made and treated by any of the methods above described (p. 115), of which Becker's is to be recommended. Others prefer to scarify the margin of the sore and examine the lymph which exudes, the flow of which may be aided by squeezing, or a small incision may be made with a very sharp knife, and then after bleeding has completely stopped to take the small drop of serum which gathers at the site. In all cases admixture of blood is to be avoided, as it interferes with the examination by the dark-ground method. It is important that neither local antiseptic applications nor any form of antisyphilitic treatment should be given before the examination is made. In the case of a lymphatic gland or non-ulcerated lesion it is best to puncture with a hypodermic needle, the point of which should be moved about in the tissue. After it is withdrawn a little saline may be placed in the syringe and pressed through the needle, the first small drop which passes, and which washes out the contents, being taken for examination; here also dark-ground illumination gives the best results.

Framboesia or Yaws

Framboesia is a disease of the tropics, occurring in the west coast of Africa, Ceylon, the West Indies, and other parts. It is characterised by a peculiar cutaneous eruption, and it is markedly contagious. Its resemblance in many respects to syphilis has been noted, and the relation of the two diseases

has been the subject of much controversy. It is accordingly a matter of great interest that an organism of closely similar characters to the Treponema pallidum has been found in the lesions of framboesia. This organism was discovered by Castellani, who gave to it the name *Spirochæta pertenuis* or *pallidula* (*Treponema pertenue castellani*). Morphologically, it is practically identical with the Treponema pallidum; when ulceration has occurred other spirochætes of less regular form may be present as contaminations. In the skin lesions it has been shown by Levaditi's method to be present in considerable numbers, especially in the epidermis and also amongst the leucocytic infiltration, which comprises more polymorphonuclear leucocytes than are seen in the case of syphilis. Castellani showed that the disease could be transferred to monkeys (*Semnopithecus* and *Macacus* being used for this purpose), and that the organism could be demonstrated in the unbroken skin lesions. The lesions are as a rule confined to the site of inoculation, but the infection is general, as is shown by the presence of spirochætes in the lymphatic glands and the spleen. These results with regard to the presence of Treponema pertenue in the lesions and the inoculation of apes have been confirmed by other workers, and the etiological relationship of the organism to the disease may now be regarded as established. Nichols has shown that a framboesia lesion can be produced in the testicle of the rabbit of similar character to the syphilitic lesion, though the period of incubation is shorter. He finds that the best means of distinguishing the two diseases is afforded by inoculating the skin of the monkey. In the case of syphilis the resulting lesion is flat, dry, and very scaly; in the case of framboesia it is elevated, slightly scaly, and very

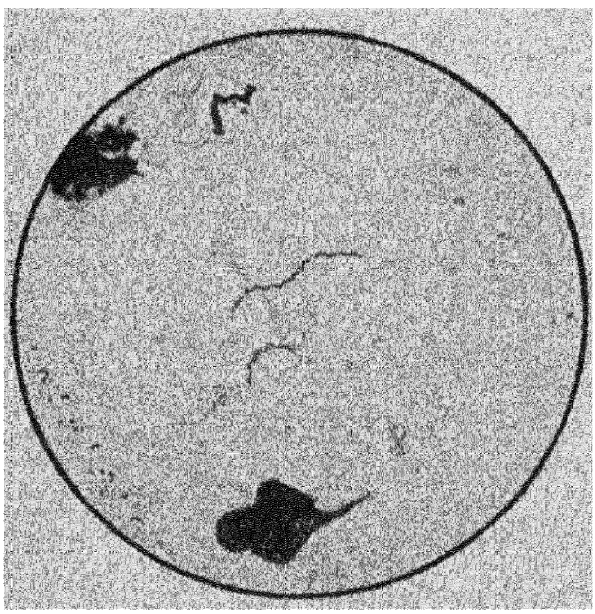


FIG. 164.—*Treponema of Yaws*. Film preparation from skin lesion in yaws, stained by Becker's method. (The organisms appear thicker than when stained by Giemsa's stain —*e.g.* Fig. 157. ×1000.

œdematous ; here also the period of incubation is shorter in the case of framboesia. The immunity reactions in monkeys infected with syphilis and framboesia, as experimentally studied by Castellani and by Neisser, Baermann, and Halberstädter, go to show that the two diseases are distinct. Nichols obtained a corresponding result in the case of the rabbit, as he found that this animal, when cured of a syphilitic lesion of the testicle by means of salvarsan, was susceptible to framboesia but not to syphilis. On the other hand, Levaditi and Nattan-Larrier found that, although monkeys infected with syphilis were refractory to framboesia, monkeys infected with framboesia were susceptible to syphilis : they therefore concluded that framboesia is a modified or mild form of syphilis. We may add that patients suffering from framboesia generally give a positive Wassermann reaction ; they are also very amenable to treatment with salvarsan (Alston and others). The exact relationship of the two diseases cannot be yet accurately defined, but they are probably distinct, though undoubtedly closely related.

CHAPTER XXIII

DISEASES DUE TO SPIROCHÆTES (*continued*): RELAPSING FEVER AND AFRICAN TICK FEVER

At a comparatively early date, namely, in 1873, when practically nothing was known with regard to the production of disease by bacteria, a highly characteristic organism was discovered by Obermeier in the blood of patients suffering from relapsing fever. This organism is now known as the *Treponema* or *Spirochæta obermeieri* (or *Borrelia recurrentis*). Obermeier described its microscopical characters, and found that its presence in the blood had a definite relation to the time of the fever, as the organism rapidly disappeared about the time of the crisis, and reappeared when a relapse occurred. His observations were fully confirmed, and his views as to its causal relationship to the disease have been established as correct.

Relapsing fever as it occurs in different parts of the world has now been carefully studied, and the relationships of the organisms have been the subject of much investigation and discussion. This question will be referred to again below. It has also been shown that the so-called "tick fever," a relapsing fever prevalent in tropical Africa, is due to a spirochæte of closely similar character. Further, it has been established that all these infections are transmitted by blood-sucking insect vectors: the European, American, and Asiatic infections by the body louse, the African type by a tick, as originally suggested by the designation "tick fever." It has also been shown that spirochætal diseases are widespread amongst vertebrates; they have been described, for example, in geese by Sacharoff, in fowls by Marchoux and Salimbeni, in oxen and sheep by Theiler, and in bats by Nicolle and Comte, and it is interesting to note that in the case of the spirochætoses of oxen and fowls the infection is transmitted by means of ticks.

The Spirochæte of Relapsing Fever in Europe, Asia, and America.—The organisms as seen in the blood during the fever are delicate spiral filaments which have a length of from 10 to

30 μ. They are, however, exceedingly thin, their thickness being 0·35–0·5 μ. They show several fairly regular coils varying in number according to the length of the organisms, and their extremities are finely pointed (Fig. 165). In some instances the coils are irregular. They are actively motile, and may be seen moving across the microscopic field with a peculiar movement which is partly twisting and partly undulatory, and disturbing the blood corpuscles in their course. There are often to be seen in the spirals, portions which are thinner and less deeply stained than the rest, and which suggest the occurrence of transverse division. Multiplication is stated to take place both by longitudinal and by transverse division.

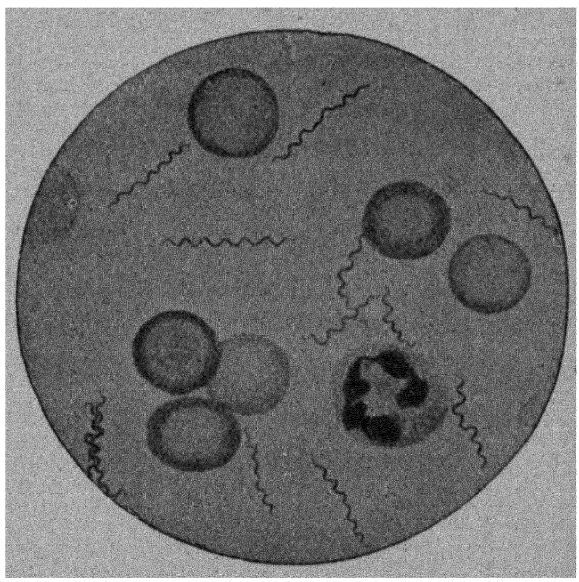


FIG. 165.—Spirochætes of relapsing fever in human blood. Film preparation. (After Koch.) See also Plate IV., Fig. 18. × about 1000.

They stain with watery solutions of the basic aniline dyes, though somewhat faintly, and are best coloured by the Romanowsky stain or one of its modifications. When thus stained they usually have a uniform appearance throughout, or may be slightly granular at places. They lose the stain in Gram's method. There is no evidence that they form spores.

Levaditi observed multiplication of Tr. duttoni (*vide infra*) in cultures made by inoculating heated serum in collodion sacs, which were then closed and placed in the peritoneal cavity of rabbits ; after repeated subcultures the organisms were still infective for animals.

Novy found that the spirochæte of American relapsing fever remained alive and virulent in defibrinated rats' blood for forty days. He also succeeded, by Levaditi's method, in obtaining cultures in collodion sacs containing rats' blood, which were placed in the peritoneum of rats. Noguchi has succeeded in cultivating the spirochætes of the various relapsing fevers by the following method. A piece of sterile tissue, *e.g.* kidney of rabbit, is placed in a test-tube ; a few drops of citrated blood from an infected animal are added and then 15 c.c. of sterile

ascitic or hydrocele fluid. The presence of a loose cogualum seems to favour growth, which occurs under anaerobic conditions. He finds that all the species multiply by longitudinal and probably also by transverse division.

Relations to the Disease.—In relapsing fever, after a period of incubation there occurs a rapid rise of temperature which lasts for about five to seven days. At the end of this time a crisis occurs, the temperature falling quickly to normal. About seven days later a sharp rise of temperature again takes place,

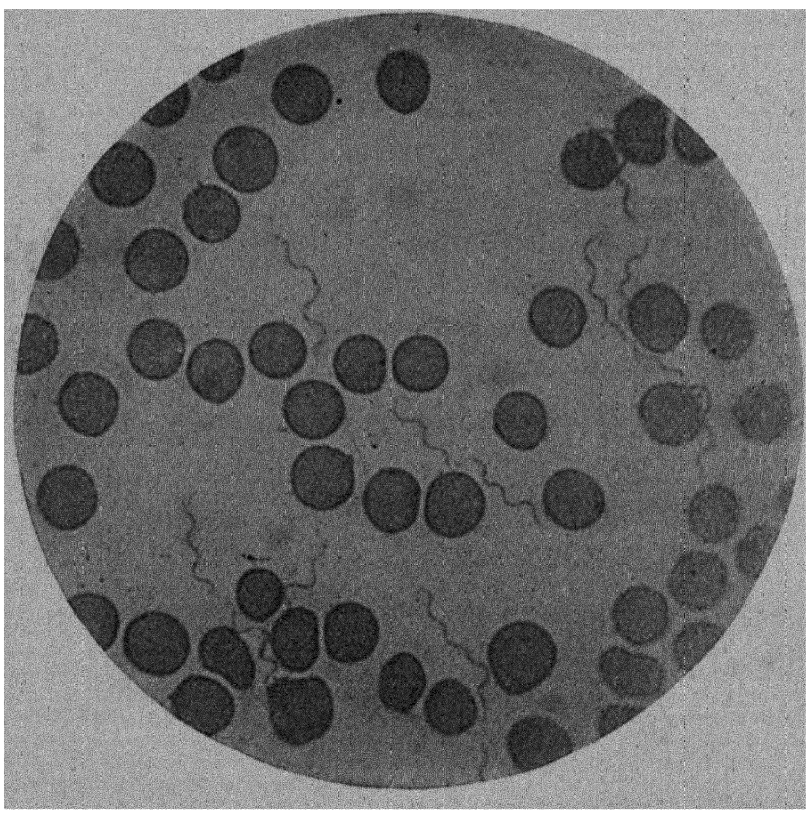


FIG. 166.—Treponema obermeieri in blood of infected mouse. ×1000.

but on this occasion the fever lasts a shorter time, again suddenly disappearing. A second or third relapse may occur after a similar interval. The organisms begin to appear in the blood shortly before the onset of the pyrexia, and during the rise of temperature rapidly increase in number. They are very numerous during the fever, a large number being often present in every field of the microscope when the blood is examined at this stage. They begin to disappear shortly before the crisis: during the afebrile interval they are entirely absent from the circulating blood. A similar relation between the presence of

the organisms in the blood and the fever is found in the case of the relapses. Münch in 1876 produced the disease in the human subject by injecting blood containing the spirochætes, and this experiment has been several times repeated with the same result. Additional proof that the organism is the cause of the disease has been afforded by experiments on animals. Carter in 1879 was the first to show that the disease could be readily produced in monkeys, and his experiments were confirmed by Koch. In such experiments the blood taken from patients and containing the spirochætes was injected subcutaneously. In the disease thus produced there is an incubation period which usually lasts about three days. At the end of that time the organisms rapidly appear in the blood, and shortly afterwards the temperature quickly rises. The period of pyrexia usually lasts for two or three days, and is followed by a marked crisis. As a rule there is no relapse, but occasionally one of short duration occurs. Norris, Pappenheimer, and Flournoy, in their experiments on monkeys with the organism of American relapsing fever, found that several relapses occurred. White mice and rats are also susceptible to infection. In the former animals the disease is characterised by several relapses; in the latter there is, however, no relapse.

Immunity.—Metchnikoff found that during the fever the spirochætes were practically never taken up by the leucocytes in the circulating blood, but that at the time of the crisis, on disappearing from the blood, they accumulated in the spleen and were ingested in large numbers by the macrophages and polymorpho-nuclear leucocytes. Within these they rapidly underwent degeneration and disappeared. It is to be noted in this connection that enlargement of the spleen is a very marked feature in relapsing fever. As in the case of so many other diseases, however, the all-important factor in the destruction of the organisms is the development of antagonistic substances in the blood. Lamb found in the case of the monkey (*Macacus radiatus*) that the immunity following an attack of the disease was due to the presence of bactericidal bodies in the serum. He found, for example, that *in vitro* the serum of an immune animal brought the movements of the spirochætes to an end, clumped them, and caused their disintegration; and further, that in one case when the spirochætes and the immune serum were injected into a fresh monkey, no disease developed. Observations by Sawtschenko and Milkich, Novy and Knapp, and Rabinowitsch, also show that in the course of infection there are developed anti-substances of the nature of immune-bodies,

with protective properties, and agglutinins. Novy and Knapp produced a "hyper-immunity" in rats by repeated injections of blood containing the spirochætes, and found that the serum of such animals had a markedly curative effect, and could cut short the disease in rats, mice, and monkeys. The course of events in the human disease may be explained by a certain degree of immunity of short duration developing during the first period of pyrexia, but some of the spirochætes in internal organs or in the tissues escape destruction by the serum or by phagocytes. These organisms then multiply and reappear in the blood, in part possibly as a result of disappearance of the immunity, but also because a strain has developed which is resistant to the protective antibodies (Levaditi and Roché). The relapse, however, is of shorter duration and less severe than the original attack, owing to the more prompt development of further antibodies which act on the relapse-strain. In the case of Indian relapsing fever, in which there is usually only one relapse, Cunningham also has shown that the strain of spirochæte present in the relapse is serologically distinct (as judged by agglutination reactions) from that observed in the original attack, and the same holds good for experimental infections; further, infection with one of these two serological types is followed by a relapse due to the other, the types present in the original attack and in the relapse alternating regularly.

Varieties.—As already stated, relapsing fever has been studied in different parts of the world, and, apart from the African relapsing fever, European, Asiatic, and American types have been distinguished. Differences have been made out with regard to clinical features, pathogenic effects, and immunity reactions. It has been shown, for example, by the work of Novy, Strong, and F. P. Mackie, that the American spirochæte is probably a distinct species, as animals immunised against it are still susceptible to infection by the European and Asiatic organisms, and *vice versa*. The relationship between the two latter is certainly closer, and no distinct immunity differences have been established. Relapsing fever in Asia is evidently a much more severe disease than in Europe; Mackie gives the mortality in Bombay at the comparatively high figure of 38 per cent. But differences in this respect, as well as in pathogenic effects, may simply depend on variations in virulence. The spirochæte of Indian relapsing fever has been designated *Tr. carteri*, that of the American infection *Tr. novyi*, while the designation *Tr. obermeieri* is retained for the European variety. Sergent and Foley have described a type of relapsing fever

occurring in Algiers, which they consider to be different from the recognised forms, and have given the name *Tr. berberum* to the organism concerned; and Balfour has observed cases in Khartoum which he thinks are probably of the same nature.

Insect Transmission.—The fact that African tick fever and certain other spirochætoses were known to be conveyed by the bites of insects suggested the probability that relapsing fever is transmitted in this way. At first the bed-bug was believed to be the vehicle of transmission, but attempts to transmit the disease by means of the bites of bugs were generally unsuccessful; F. P. Mackie produced the disease in only one out of six monkeys used for this purpose, though large numbers of bugs, which had bitten relapsing fever patients, were used. On investigating an epidemic of the disease, however, he obtained a considerable amount of evidence on epidemiological grounds that the disease was carried by the body louse. He also found that the spirochætes in the blood which had been ingested underwent great multiplication about three days afterwards, and formed large tangled masses in the stomach contents. The view that the louse is the agent of transmission of the human disease is strongly supported by the work of Nicolle, Blaizot, and Conseil, and by the experiments of Manteufel, who was able to transmit infection from rat to rat in nearly 60 per cent. of the experiments made, whereas he obtained only negative results by means of bugs. The mode of inoculation of the organism from infected lice is by its introduction into abrasions produced by scratching—the excreta of the insect or the crushed insect providing the inoculum.

African Relapsing Fever

The disease long known by the name of Tick Fever as prevalent in Tropical Africa has also been shown to be caused by a spirochæte—*Tr. duttoni*. Organisms of this nature had been seen in the blood of patients in Uganda by Greig and Nabarro in 1903, and Milne and Ross in 1904 recorded a series of observations which led them to the conclusion that tick fever was due to a spirochæte. It is, however, chiefly owing to the work of Dutton and Todd in the Congo Free State, on the one hand, and of Koch in German East Africa, on the other, that our knowledge of the etiology of the disease has been obtained.

The following are the chief facts regarding this disease. Clinically, the fever closely resembles relapsing fever, but the periods of fever are somewhat shorter, rarely lasting for more

than two or three days, and the relapses are more numerous. It is seldom attended by a fatal result unless in patients debilitated from other causes. The organisms in the blood are considerably fewer than in the case of European relapsing fever, and sometimes a careful search may be necessary before they are found. Morphologically, they are said to be practically identical, although Koch thought that the organisms in tick fever tended on the whole to be slightly longer; the average length may be said to be 15–35 μ (Figs. 167, 168). Dutton

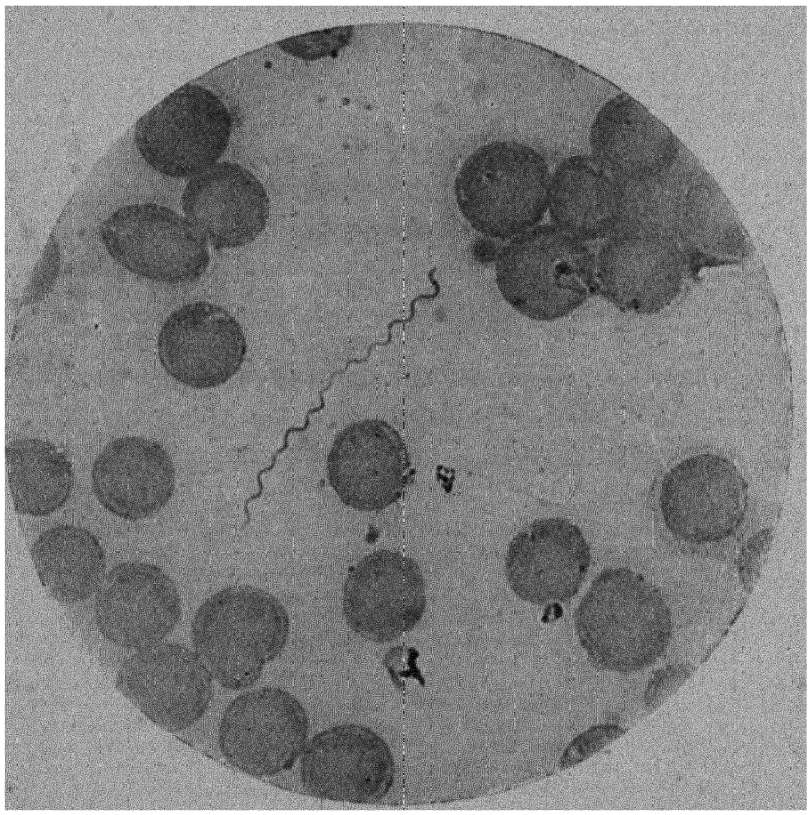


FIG. 167.—Film of human blood containing spirochæte of tick fever. ×1000.[1]

and Todd showed that it was possible to transmit the disease to certain monkeys (*Cercopitheci*) by means of ticks which had been allowed to bite patients suffering from the disease, the symptoms in these animals appearing about five days after inoculation. The disease thus produced is characterised by several relapses, and often leads to a fatal result. In one case they produced the disease by means of young ticks hatched from the eggs of ticks which had been allowed to suck the blood

[1] We are indebted to the late Lt.-Gen. Sir William Leishman, R.A.M.C., for the preparations from which Figs. 166–168 were taken.

of infected patients, and they came to the conclusion that the spirochætes were not simply carried mechanically by the ticks, but probably underwent some cycle of development in the tissues of the latter. Leishman has since shown that the ticks of the second generation may also be infectious. The species of tick concerned is the *Ornithodorus moubata*. These results were confirmed and extended by Koch. He found that after the ticks had been allowed to suck the blood containing the organisms, these could be found for a day or two in the stomach

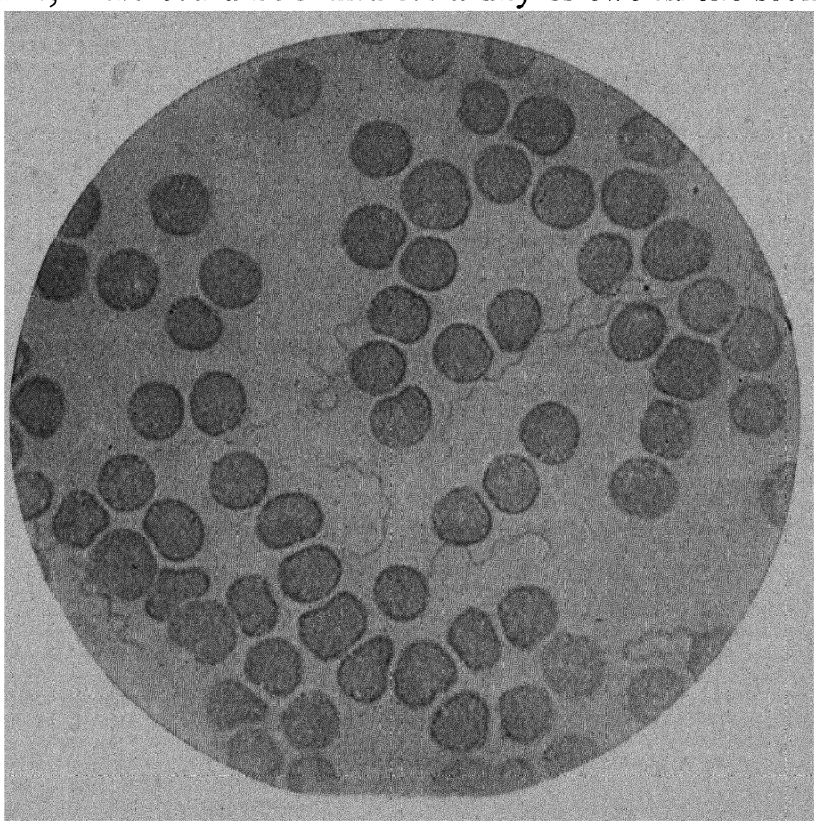


FIG. 168.—Spirochæte of human tick fever (Tr. duttoni) in blood of infected mouse. ×1000.

of the insect. After this time they gradually disappeared from the stomach, but were detected in large numbers in the ovaries of the female ticks, where they sometimes formed felted masses. He also traced the presence of the spirochætes in the eggs laid by the infected ticks, and in the young embryos hatched from them. On the other hand, Leishman failed to find any evidence of spirochætes in the tissues of ticks later than ten days after ingestion of infected blood, or in the ova laid by the ticks, or in the young ticks when hatched, though these were proved by experiment to be infective. After ingestion of the blood

by the ticks, he found that morphological changes occur in the spirochætes, resulting in the formation of minute "chromatin granules" which are extruded and traverse the walls of the intestine and are taken up by the cells of the Malpighian tubules; they also penetrate the ovaries and may be found in large numbers within the ova. Similar granules are to be seen in the Malpighian tubules of the embryo ticks, where they are also found in the subsequent stages of their life. He proved that infection of animals might be produced by inoculation with crushed material containing the granules but no spirochætes. He accordingly considered that the granules in question represent a phase in the life-history of the parasite, and that infection occurs by inoculation of the skin with the chromatin granules voided in the Malpighian secretion and not by unaltered spirochætes from the salivary glands. A similar view is taken by Hindle, who found that when infected ticks, in which the spirochætes had disappeared, were heated to a temperature of 35° C., the spirochætes reappeared in the organs and cœlomic fluid. It is also interesting to note that Balfour found similar granules in ticks (*Argas persicus*) infected with *Treponema gallinarum,* and he also observed the formation of granules from spirochætes in the blood of Sudanese fowls treated with salvarsan.

Koch also made extensive observations on the ticks in German East Africa, and found that of over six hundred examined along the main caravan routes, 11 per cent. contained spirochætes, and in some localities almost half of the ticks were infected. In places removed from the main lines of commerce he still found them, though in smaller number. It has also been demonstrated that in some places the ticks are found to be infected with the spirochætes although the inhabitants do not suffer from tick fever, a circumstance which is probably due to their having acquired immunity against the disease.

It is now generally believed that the Tr. duttoni is a species distinct from, though closely allied to, the organisms of the relapsing fevers, described above. We have mentioned some differences in the clinical characters of the diseases, and there are also differences in the pathogenic effects of the organisms on inoculation. The Tr. duttoni, for example, produces a much more severe disease in monkeys, and is pathogenic to more species of the laboratory animals than the Tr. obermeieri. The most important differences are, however, brought out by immunity reactions. It was shown by Breinl that the immunity produced by the Tr. obermeieri did not protect against the Tr.

duttoni, and that the converse also held good; and it has since been established that a similar difference obtains between the Tr. duttoni and the organisms of the Asiatic and American varieties of relapsing fever. Corresponding results are obtained on testing the various serum reactions *in vitro*. As already stated, Noguchi has cultivated the Tr. duttoni artificially, and from a study of its characters agrees that it is a distinct species.

Spirochætal or Infective Jaundice

This affection, long known as Weil's disease, was proved in 1915 by Inada and other Japanese workers to be due to a spirochæte, to which they gave the name *Spirochæta icterohæmorrhagiæ* (*Leptospira icterohæmorrhagiæ*). This spirochæte is characterised by its exceedingly numerous and fine closely wound coils, and in 1917 Noguchi applied to it the generic name of *Leptospira* on account of its morphology. The pathology of the condition has now been carefully studied by many workers. The disease is characterised by the sudden onset of symptoms, general malaise, pyrexia lasting about ten days, a tendency to hæmorrhage from mucous surfaces, conjunctival congestion, hæmorrhagic herpes, and jaundice which becomes increasingly marked from the fourth day of the illness. The occurrence of the disease in small epidemics had been previously noted. In Japan it was found to occur amongst workers in the same part of wet mines. It occurred during the war amongst the troops in France, and the results of the Japanese workers were confirmed by bacteriologists in both the British and French armies. It was also found on the Italian front and amongst the German troops. The infection had thus a wide distribution during the war, but the mortality was much lower than that met with in Japan. More recently the disease has been described by Gulland and Buchanan among coal miners working in wet mines in Scotland, and the mortality so far observed has been practically the same as in Japan.

Morphology of the Spirochæte.—The organism in the blood and tissues measures 6–9 μ in length (but both shorter and longer forms occur) and about 0·25 μ in thickness; that is, it is a slender organism of about the thickness of the Tr. pallidum. In cultures it may grow into much longer threads. It is somewhat thicker in the middle and tapers towards the ends, which may be pointed, but there are no terminal flagella (Fig. 169). The morphology is best demonstrated by dark-ground illumination and is characterised by numerous fine elementary spirals and

incurved or "hooked" ends, while occasionally secondary spirals

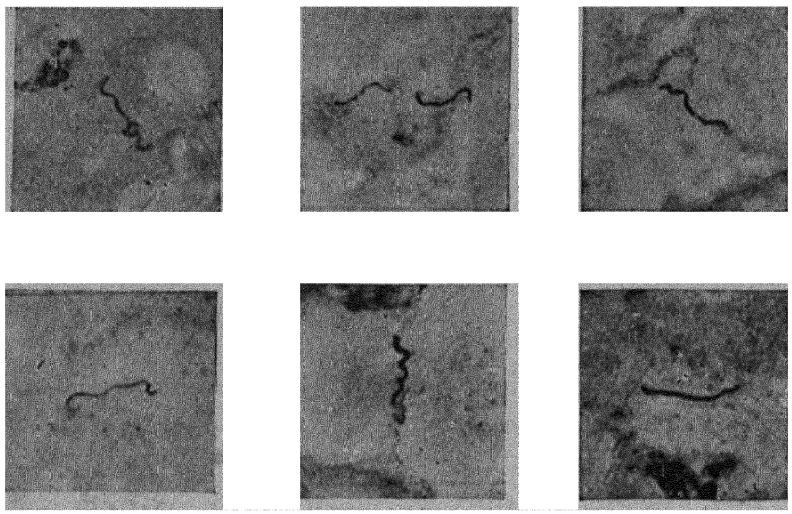


FIG. 169.—Specimens of Leptospira icterohæmorrhagiæ, as seen in sections of a suprarenal of an infected guinea-pig. Stained by Levaditi's method.
(From a preparation by Major J. W. M'Nee, R.A.M.C.) ×1000.

or undulations are observed (Fig. 170). In stained preparations the elementary spirals are not easily demonstrable (Fig. 169). It is actively motile, the movements being rotatory and undulatory. It can be studied by all the microscopic methods already described in the case of the Tr. pallidum (p. 573).

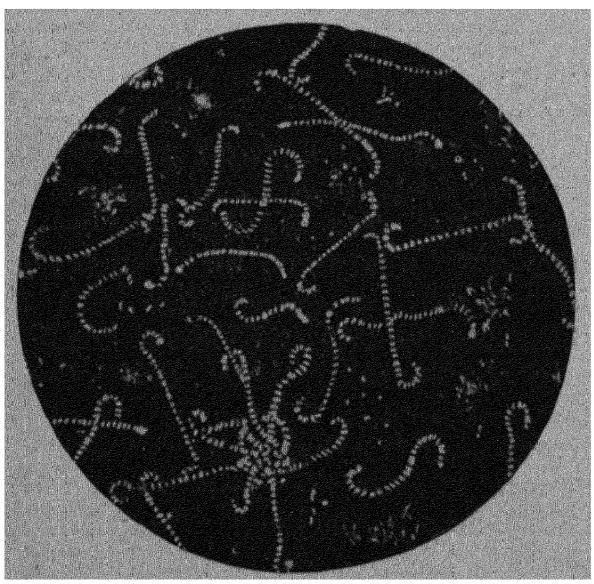


FIG. 170.—L. icterohæmorrhagiæ, as seen in a culture by dark-ground illumination, showing elementary spirals. (From a drawing by Dr. Geo. Buchanan.)

Cultivation. — The organism was first successfully cultivated in Noguchi's medium for Tr. pallidum, in which the initial growth survived for three to six weeks. Later it was grown in solid media, blood agar and blood gelatin, the former being the more suitable.

The limits of growth are wide, namely, 13°–37° C., the optimum temperature being 30°–37° C. The following media are suitable:

Noguchi's Medium.—(*a*) A mixture of rabbit's serum, 2 parts, Ringer's solution (or 0·9 per cent NaCl solution), 6 parts, citrated rabbit's plasma (or sterile defibrinated rabbit's blood), 1 part. (*b*) The same with the addition of 1–2 parts of neutral or slightly alkaline 2 per cent. agar, which should be melted and added when at a temperature of 60°–65° C. in order to get a uniform mixture. Both media are covered with a layer of sterile liquid paraffin, and inoculation is made through the paraffin. The above are most suitable for primary cultures. For subcultures one may use Wenyon's modification prepared as follows: To 270 c.c. of 0·85 per cent. sodium chloride solution (P_H 7·6) are added 30 c.c. of ordinary 2 per cent. bacteriological nutrient agar (P_H 7·6). When mixture has taken place, 10 c.c. are placed in each test-tube. After autoclaving at 120° C. the tubes are cooled to 50° C. and into each tube are allowed to drop, from a rabbit's ear, 20 drops of blood under aseptic precautions (see p. 53). The tubes are not shaken or mixed, and are incubated for twenty-four hours at 37° C. The medium is then ready for use. In such media growth produces a visible opacity in the top layer. Most abundant growth is obtained when medium is disposed as a thin layer in flat-bottomed flasks.

Relations to the Disease.—The organism occurs both in the blood and in the organs. In the former it is found in the first four or five days of the disease; thereafter it gradually disappears, and in the second week, when jaundice is most marked, it cannot be detected. The best method of demonstrating its presence is to draw off some blood, say 3–5 c.c., and inject it into the peritoneal cavity of the guinea-pig, in which animal it produces an infection and can easily be found (*vide infra*). It is rarely present in the blood in the human subject in numbers sufficient to allow its detection by microscopic examination.

Of the internal organs the liver contains the organisms in largest quantities; they may be also found in the suprarenals, and, especially at a later stage, in the kidneys. In all the organs in the human subject the spirochætes are scanty, they are often somewhat irregular and degenerated in appearance, and often occur in the interior of the special cells and in the interstitial tissue. These facts have been explained as being the result of the formation of anti-substances, which drive them from the blood into the interstitial tissues. Their late occurrence and persistence for some time in the kidneys are comparable with what occurs in the natural infection of the rat without the occurrence of disease symptoms (*vide infra*). The spirochæte is also excreted in the urine. This does not occur in the earliest stage of the disease, but from about the tenth day onwards

positive results are obtained in increasing numbers, till about the twentieth day it may be found in practically all cases. Thereafter it gradually disappears and is rarely found after the fortieth day. The best methods are to examine by dark-ground illumination the deposit thrown down from the urine by a high-speed centrifuge and at the same time to inoculate a guinea-pig intraperitoneally.

The gradual development of antibodies in the blood has been shown to occur during the disease. These appear towards the end of the first week, and seem to be related to the disappearance of the organism from the blood; they become specially marked during the second week. Their presence can be demonstrated by injecting some of the patient's serum along with the spirochætes into a guinea-pig, death being thus prevented, or at least the onset of the illness being postponed. Destruction of the organisms under the influence of the anti-serum may be observed in some cases microscopically in the peritoneal cavity of the animal, that is, spirochætolysis occurs, corresponding to Pfeiffer's phenomenon in the case of bacteria.

Experimental Inoculation.—The injection of blood or of emulsions of organs containing the spirochætes into the peritoneal cavity of a guinea-pig leads to an infection which is usually fatal in about seven to twelve days; the same holds with regard to the effect of pure cultures. The symptoms are conjunctival congestion, jaundice, tendency to hæmorrhages, albuminuria, and anæmia. There is pyrexia, which towards the end is succeeded by subnormal temperature; the jaundice becomes visible in the integuments about the third or fourth day and increases up to the time of death. Spirochætes usually appear in the peripheral blood about the fourth day of the illness and increase in numbers until the animal succumbs. *Post mortem,* the subcutaneous tissues are usually intensely jaundiced; hæmorrhages may be generalised or they may be confined to the lungs, intestinal walls, and retro-peritoneal tissue; acute parenchymatous or hæmorrhagic nephritis is present, and the spleen is large and congested. The hæmorrhages in the lungs occur as small and large spots, described as being "like the wing of a mottled butterfly." The spirochætes are present in the blood and organs, and in the latter are chiefly interstitial in position, few being actually within cells. In this respect there is a difference from what obtains in the human disease. They are most abundant in the liver, where they may be arranged like a garland round the liver cells. The adrenals and the kidneys contain considerable numbers, but they are scanty in

the spleen, bone-marrow, and lymphatic glands. The Japanese workers believed that, in the human disease, infection occurs chiefly through the alimentary tract, and they were able to produce the disease in the guinea-pig by feeding with material containing the organism or by introducing some of it into the rectum. Händel, Ungermann and Jaenisch, and Buchanan, however, state that infection cannot be produced experimentally in animals by feeding. The Japanese observers showed that infection could take place through the apparently intact skin, and found that this occurred with comparative rapidity, as the application of an antiseptic five minutes after the infective material did not prevent infection. The occurrence of infection through the skin has been confirmed by Buchanan.

A highly important point with regard to the epidemiology of the disease is the common presence of the spirochæte in both house and field rats and field mice without any apparent disturbance of health. The occurrence of the L. icterohæmorrhagiæ in these wild rodents in practically all parts of the world has now been well established, and the proportion of infected rats may be relatively high, *e.g.* in Scotland 36 per cent. (Buchanan). The organisms are practically confined to the kidneys, and we have here a resemblance to what is found in the human infection at a later stage when immune-substances are present in the blood. The spirochætes are passed in large numbers in the urine of the infected animals, and in this way contamination of the soil and various articles is brought about. The spirochætes obtained from rats are stated to vary considerably in virulence for experimental animals, but this apparent variation in virulence may be due to varying susceptibility among the animals. An interesting contribution to our knowledge of the epidemiology of infectious jaundice is due to Buchanan, who has demonstrated a pathogenic strain of Leptospira icterohæmorrhagiæ in the roof slime of a wet coal mine in Scotland, from which an outbreak of infective jaundice had originated. The disease occurred in miners working in the particular part of the mine in which the organisms were found. Buchanan has suggested that the leptospira of infective jaundice may exist as a saprophyte under natural conditions, and from a natural source infect animals or man. Non-pathogenic spirochætes similar to L. icterohæmorrhagiæ have also been observed in water supplies, mines, etc., by various workers. Further investigation is still required as to the biological and pathological relationship of these saprophytic strains.

It may be noted here that an infectious jaundice of dogs

known as " yellows," has recently been shown to be due to *L. icterohæmorrhagiæ*, and the condition in these animals corresponds to the human disease.

Therapeutic antisera to the *L. icterohæmorrhagiæ*, with properties similar to those observed in the serum during the disease (*vide supra*), have been prepared and have proved of value in the treatment of cases.

Yellow Fever

Yellow fever is an infective disease which is endemic in the West Indies, in Brazil, in Sierra Leone and the adjacent parts of West Africa. From time to time serious outbreaks take place, during which neighbouring countries also suffer, and the disease may spread to other parts of the world. In this way epidemics have arisen in the United States and elsewhere. In the parts where it is endemic, though usually a few cases may occur from time to time, there is some evidence that occasionally the disease may remain in abeyance for many years and then originate apparently *de novo*. It is possible that continuity may be maintained by the persistence of a mild type of the disease, which may be grouped with the " bilious fevers " prevalent in yellow fever regions. This would explain the degree of immunity which is shown during a serious epidemic by the older inhabitants.

Great variations are observed in the clinical types under which the disease presents itself. Usually after from two to six days' incubation a sudden onset in the form of a rigor occurs. The temperature rises to 104°–105° F. The person is livid, with outstanding bloodshot eyes. There are present great prostration, pain in the back, and vomiting, at first of mucus, later of bile. The urine is diminished and contains albumin. About the fifth day an apparent improvement takes place, and this may lead on to recovery. Frequently, however, the remission, which may last from a few hours to two days, is followed by an aggravation of all the symptoms. The temperature rises, jaundice is observed, hæmorrhages occur from all the mucous surfaces, causing, in the case of the stomach, the " black vomit " —one of the clinical signs of the disease in its worst form. Anuria, coma, and cardiac collapse usher in a fatal issue. The mortality varies in different epidemics from about 35 to 99 per cent. of those attacked. Both white and black races are susceptible, but those who have resided long in a country are

less susceptible than new immigrants. An attack of the disease usually confers complete immunity against subsequent infection.

Post mortem the stomach is found in a state of acute gastritis, and contains much altered blood derived from hæmorrhages which have occurred in the mucous and submucous coats. The intestine may be normal, but is often congested and may be ulcerated; the mesenteric glands are enlarged. The liver is in a state of fatty degeneration of greater or less degree, but often resembling the condition found in phosphorus poisoning. The kidneys are in a state of intense glomerulo-nephritis, with fatty degeneration of the epithelium. There is congestion of the meninges, especially in the lumbar region, and hæmorrhages may occur. The other organs do not show much change, though small hæmorrhages under the skin and into all the tissues of the body are not infrequent. In the blood a feature is the excess of urea present, amounting, it may be, to nearly 4 per cent.

Etiology of Yellow Fever.—The earliest bacteriological work on the etiology of yellow fever is now only of historical interest. Long before the actual virus of the infection came to be investigated, it was suspected that a mosquito acted as the intermediary host, and in 1881 Finlay claimed that *Stegomyia fasciata* (now designated *Aëdes calopus*) was the vector of the infection. These early views on the transmission of the disease were confirmed by later investigations. Further, the facts elicited in regard to transmission by mosquitoes suggested that the causal organism underwent some phase in its cycle of development in the body of the insect.

In 1900 a United States Army Commission commenced a careful investigation into the etiology of the disease, and the data obtained proved of the utmost importance. The members of the Commission first directed their inquiries towards determining whether the Bacillus icteroides,[1] described by Sanarelli in 1897 as the causal agent, was present in the blood during life, and a series of cases was investigated bacteriologically, with entirely negative results in each instance. They then resolved to test the hypothesis of Finlay, that the disease was carried by mosquitoes. Selecting mosquitoes which they had reared from eggs, they allowed them to bite yellow fever patients and then to bite healthy men. Of several experiments of this nature two were successful in the first instance, the first individual to be infected in this way being Dr. James Carroll, a member of the Commission, who passed through a severe attack of typical

[1] A type of organism biologically related to the paratyphoid group.

yellow fever. Experiments were then performed on a larger scale, with completely confirmatory results as to the conveyance of the disease by mosquitoes. Of twelve non-immunes living under circumstances which excluded natural means of infection, ten contracted yellow fever after having been bitten by mosquitoes which had previously bitten yellow fever patients; happily all of these recovered. Two of the men who were thus infected had been previously exposed to contact with fomites from yellow fever patients without results. These results were confirmed by Guitéras, whose investigations were carried out along similar lines; of seventeen individuals bitten by infected mosquitoes, eight took yellow fever, and three of these died.

The species of mosquito used by the American Commission was the *Aëdes calopus* (*Stegomyia fasciata*), and up to the present time no other species has been found capable of carrying the infection. It has also been determined that a certain period must elapse after the insect has bitten a yellow fever patient before it becomes infective to another subject. In summer weather this period is about twelve days; at a lower temperature somewhat longer. This suggests that, as in the case of malaria, the parasite must pass through certain stages of development before it reaches the salivary gland and is thus in a position to be transferred to a fresh subject. Infected mosquitoes, however, retain the power of infection for a considerable time afterwards, probably as long as sixty days. It has also been shown that mosquitoes may become infective after biting a patient on the first, second, or third day of the disease, but at a later period the results are usually negative, apparently because the virus is no longer present in the blood.

Interesting results were also obtained with regard to the communication of the disease directly from patient to patient, the conclusion arrived at, after careful experiments, being that the disease cannot be transferred in this way, even when the contact is of a close character. In a specially constructed house seven men were exposed to the most intimate contact with the fomites of yellow fever patients for a period of twenty days each, the soiled garments worn by the patients being in some cases actually slept in by these men; the result was that not one of those thus exposed contracted the disease. The conclusions on this point have been subsequently confirmed by other workers.

The American Commission also found it possible to transmit yellow fever to a healthy man by injecting small quantities of blood or of serum taken from a yellow fever patient at any

period up till the third day of the disease. The period of incubation in this case is somewhat shorter than when the disease is conveyed by the bite of mosquitoes, the average duration in the former case being about three days, and in the latter about four days, though these times may be considerably exceeded. It is also interesting to know that the blood or serum used in these experimental infections was found to be free from bacteria. Up till the present time, we know of only these two methods of infection, namely, indirectly by the bite of a mosquito infected with the yellow fever virus; or directly by the injection of some of the blood from a yellow fever patient. It has been stated that it is possible to produce yellow fever in the chimpanzee by the injection of blood from a patient.

Experiments with regard to the nature of the yellow fever organism were carried out by Reed and Carroll, and interesting results were obtained. They found that the organism of the disease was very easily killed by heat, as blood from a yellow fever patient lost its infective power on being heated to 55° C. for ten minutes. On the other hand, blood or serum was found to be still infective after having been passed through a Berkefeld filter. This was confirmed by the French Commission, with the additional result that the virus passed through a Chamberland F filter, but not through a Chamberland B. These facts led to the classification of the parasite among the so-called ultramicroscopic or filterable viruses, and attempts to demonstrate the organism by microscopic methods failed, though in 1912 Seidelin claimed to have discovered an intracorpuscular parasite resembling a piroplasm. His observations were not, however, confirmed.

Though until 1918 nothing had been determined regarding the actual nature of the virus, yet the results obtained supplied the basis for preventive measures against the disease, these being directed towards the destruction of mosquitoes and the protection of those suffering from yellow fever, and also the healthy, against the bites of these insects. A striking degree of success was met with in Havana. Such measures came into force in February 1901, and in ninety days the town was free of yellow fever, and for fifty-four days later no new cases occurred; and although subsequently the disease was reintroduced into the town, no difficulty was experienced in stamping it out by the same measures. Later results also proved highly gratifying, and the disease may be said to be practically eradicated from Havana. In other large centres of population, for example Rio de Janeiro, equally successful results were obtained,

and epidemics in limited areas would appear to be now under control if the proper measures are taken. In striking contrast to this is the fact that in certain places where preventive measures were not satisfactorily instituted, owing to the population being scattered or other causes, the mortality from yellow fever still remained high.

In 1918, a Commission was sent to Guayaquil, in Ecuador, by the International Health Board of the Rockefeller Foundation, for the further investigation of the disease, and the etiological study was undertaken by Noguchi, who elicited important new data and claimed to have demonstrated a leptospira similar to L. icterohæmorrhagiæ (*vide* p. 585) as the causal organism, which he designated *L. icteroides*. Injection of the blood from yellow fever cases into young guinea-pigs produced, in nearly 25 per cent. of the experiments, a condition of fever, jaundice, nephritis, and hæmorrhages in the gastro-intestinal tract and in other viscera. Sometimes a positive result was obtained only after the blood had been subjected to preliminary enrichment in culture media. The experimental disease thus produced resembled yellow fever in man. In the blood and in the liver and kidneys of the experimental animals, a leptospira was demonstrated microscopically, and cultivated artificially by the methods used for the L. icterohæmorrhagiæ (*vide* p. 587), and the disease was transmitted to guinea-pigs by inoculation with pure cultures. The microscopic demonstration of the organism in the blood and organs of the human disease was, however, inconstant. The leptospira proved to be a filter-passer, and Noguchi's results thus fell into line with those of the previous Commission. The experimental infection, as in the case of the actual disease, was found to produce a permanent immunity, due to serum antibodies demonstrable by the Pfeiffer reaction. The leptospira was also observed by dark-ground illumination in infective mosquitoes, and guinea-pigs could be infected by the injection of emulsions of these insects. Noguchi's original results were confirmed in subsequent years by other observers, and Iglesias experimentally transmitted the infection in animals by mosquitoes. Apparently a difficulty in the experimental work on yellow fever depends on the varying susceptibility of guinea-pigs to the organism, and, further, the leptospira is only present in the blood of patients during the first three to five days of the disease. The question has also been studied as to the relationship of the L. icteroides to the L. icterohæmorrhagiæ. There is no essential difference in morphology between the two organisms, but Noguchi has pointed out differences in their immunological

and pathogenic properties. Thus, L. icteroides is markedly "icteronephritic," and the infection is specially characterised by fatty degeneration of the parenchyma of the liver and kidney and hæmorrhages in mucous membranes, while in L. icterohæmorrhagiæ infection fatty degeneration of the liver and kidney is not a characteristic feature, and hæmorrhages occur more frequently in serous membranes, subcutaneous tissues, muscles, lungs, etc., than in mucous membranes (*vide* p. 588). It must be noted that while yellow fever and infectious jaundice present marked differences in their epidemiological features and in severity and mortality rate, there is a substantial correspondence between their clinical and pathological characters, and borderline cases may be met with which can only be diagnosed on epidemiological grounds. According to Noguchi, immunological and serological reactions serve to differentiate the two organisms. Killed cultures of L. icteroides have been used as a vaccine for prophylactic inoculation with encouraging results, and an antiserum obtained by artificial immunisation of animals has also been applied in treatment, with a reduction in mortality among patients treated during the first four days of the illness as compared with that among the non-treated.

Rat-bite Fever

This condition, following the bite of a rat after an incubation period of ten to twenty-two days, presents a characteristic clinical syndrome :—Inflammation of the skin in the neighbourhood of the bite, which may have completely healed by the time of onset of the disease, paroxysms of fever of the relapsing type, swelling of lymph glands, and a patchy erythematous skin eruption. The disease has been observed in various parts of the world, but has been most studied in Japan, and in 1915 Futaki and his co-workers described a special type of spiral organism in the skin lesion and in the lymph glands, which has now been well established to be the specific causal agent of the disease. They regarded it as a spirochæte and it was designated *Spirochæta morsus muris*. Previous to this, Schottmüller and also Blake described streptothrix-like organisms isolated from the blood in cases of rat-bite fever presenting the characteristic clinical manifestations ; but it seems probable that these organisms may have represented, in the particular cases, a concomitant or secondary infection.

The causal organism occurs in the local lesion, the related lymph glands, and in some cases in the blood. It is comparatively short, measuring 2–5 μ, and presents a few regular curves of 1 μ each (Fig. 171) ; it is relatively thick and easily stained, especially by means of a Romanowsky stain. By dark-ground illumination it is most readily demonstrated, *e.g.* in the blood of an infected animal, and exhibits very active motility of a "darting" type similar to that of a vibrio. Polar flagella (1–7) are present at each

end ; they are more frequently multiple than single. The organism is practically non-flexuous and in its biological characters conforms more to a spirillum than to a spirochæte. It has been cultivated artificially by Futaki and co-workers in Shmamine's medium prepared as follows : 0·5 to 0·75 grm. sodium nucleate is dissolved in 100 c.c. horse serum ; carbon dioxide is passed through the solution for four minutes, and the medium is then heated on three successive days for an hour at 60° C. ; on the fourth day, it is again heated at 65° C. for thirty minutes, when it undergoes partial coagulation. Other observers have failed to obtain cultures, however, in various media, and the Japanese workers were unable to maintain their growths after subculture.

This organism has now been demonstrated in a few cases of rat-bite fever in this country. Recently Mackie investigated a case occurring in Scotland : the patient presented the typical clinical condition and the characteristic spirillum was demonstrated by

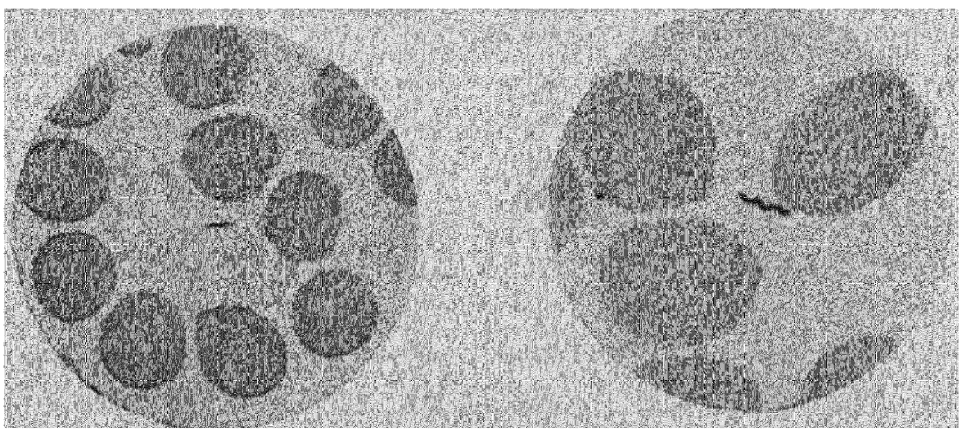


FIG. 171.—Sp. morsus muris, as seen in blood films from experimentally infected guinea-pig. A × 1000. B × 2000.

inoculation of guinea-pigs with an emulsion of an excised regional lymph gland (*vide infra*). It is interesting to note that a condition similar to rat-bite fever has followed the bites of cats and ferrets. Rats, mice, and guinea-pigs can be infected with the spirillum of rat-bite fever, and the organism appears in their peripheral blood. Its virulence to laboratory animals is relatively low, though guinea-pigs are fairly susceptible and, after a time, succumb to the infection. Occasionally individual guinea-pigs are resistant. Mice usually become refractory to the infection after the initial attack, and the organisms practically disappear from the blood. The spirillum occurs in a small percentage of rats and field mice under natural conditions, and the disease has been produced in the guinea-pig from the bite of an infected rat. The mechanism of infection is doubtful. It has been suggested that when the animal bites, bleeding from the mouth provides the material for inoculation. Infected animals may develop a keratitis with spirilla in the conjunctival secretion, and from this the nasal and mouth secretion becomes infected. The infection is highly

amenable to treatment by salvarsan. It has been shown also that the blood of a convalescent patient possesses protective antibodies.[1]

For diagnostic purposes, 3 to 5 c.c. of blood withdrawn during the pyrexial stage can be injected intra-peritoneally into laboratory animals, but this method may fail, and it is advisable to puncture an enlarged gland with a syringe, aspirate some fluid, and inject a guinea-pig and a white rat. After a varying incubation period, the spirillum can be demonstrated, by the methods referred to above, in the peripheral blood of the infected animals. The blood and gland-juice may also be examined directly. Excision of a gland and inoculation of animals with the emulsified tissue provides the most certain method of demonstrating the spirillum. In some cases the organisms may be demonstrated directly in the local lesion, and the exudate from the lesion may also be used for animal inoculation tests.

Phlebotomus Fever

In the Adriatic littoral there occurs a disease known as "pappataci," characterised by fever and pains in the muscles lasting for about three days, followed by somewhat prolonged prostration, but very rarely having a fatal issue. Doerr, after failing to isolate any organism from the blood, found that the subcutaneous injection of from 0·5 to 1 c.c. of the serum from a case (during the first day of the illness) into a healthy individual was followed about eight days later by an attack of the disease. A similar effect was produced with the serum after it had been passed through a Berkefeld filter—all the inoculation experiments being performed at a distance from the original location of the disease. The view was therefore put forward that the causal agent belonged to the category of the ultra-microscopic viruses. Owing to the prevalence of the disease during the summer season Doerr considered there was justification for the popular view that it was associated with the bite of the dipterous fly, *Phlebotomus pappatasii*. This was borne out by the fact that on feeding such flies on a sick person, transporting them to a locality free from the disease and allowing them to bite healthy individuals, the affection was reproduced. An apparently identical disease occurs in Malta, and was first investigated by Birt under the name of "Phlebotomus Fever." This observer fully confirmed Doerr's results, the condition again being reproduced by infected flies, which, however, were found not to manifest infectivity earlier than seven days after biting. This last fact appeared to indicate that the causal

[1] A somewhat similar organism, *Sp. muris* (or *Sp. laverani*), occurs in the blood of mice; it is non-pathogenic for these animals and has not been stated to flourish in other species.

organism has a developmental cycle in the fly. Birt found that the virus could pass through a Chamberland "F" filter. The disease also occurs in India and Mesopotamia, in Northern Africa, in Corsica, in Calabria, and probably generally throughout Italy, in Portugal, and in Arabia.

In recent years additional light has been thrown on the etiology of the disease by the work of the Royal Air Force Sandfly Fever Commission, who demonstrated the presence of spirochætes of the leptospira type in six cases in Malta. The organisms were isolated by blood cultures in a medium similar to that used for the L. icterohæmorrhagiæ (*vide* p. 587) and appeared in the cultures after five to six days' incubation at 27° C. They proved morphologically identical with L. icterohæmorrhagiæ, but were non-pathogenic to guinea-pigs. This Commission also carried out extensive experiments on the infectivity of the sandfly, infectivity being determined by the occurrence of the characteristic fever in volunteers who had been bitten by captive flies. It was found that in an endemic area all phlebotomi are not infected with the virus and that the infection of the insect is not hereditary, but the experiments indicated that the infection can be transmitted to insects in their breeding-grounds by the larvæ ingesting the dejecta or dead remains of the adult flies (Whittingham). It was also noted that atmospheric temperature influenced the infectivity of the flies; thus insects, proved to be infective when the temperature was above 65° F., became non-infective after a fall below 60° F.

Phlebotomus fever may be regarded as an example of a group of similar affections occurring in various tropical and subtropical parts of the world—chiefly in coastal areas—and going under a variety of names. This group includes the seven-day fever of Eastern countries, dengue, and the three-day fevers of various regions (*e.g.* the so-called Canary fever, Shanghai fever, Chitral fever).

Seven-day Fever

Seven-day fever has been investigated recently by Ido, Ito and Wani in Japan, who have established the constant presence in the disease of a leptospira which has been designated *L. hebdomadis*. The organism is morphologically similar to *L. icterohæmorrhagiæ*, and in some cases can be demonstrated by appropriate methods in the peripheral blood, though not abundant. As in infectious jaundice, the leptospira is discharged in the urine, often in large numbers, and especially in

the convalescent stage. The spirochæte is pathogenic to guinea-pigs under 200 grams in weight, but larger guinea-pigs are relatively insusceptible. In experimental animals a febrile condition is produced, but icterus is only occasionally observed and, when present, is slight, and there is less tendency to hæmorrhage. The organism can be detected in the blood and *post mortem* in the liver and kidneys. The experimental disease contrasts with that due to L. icterohæmorrhagiæ, though there may be some similarity. Infected animals may also recover from the disease. It is claimed by the Japanese workers that this organism constitutes a species distinct from L. icterohæmorrhagiæ, not only on the basis of pathogenic effects but also in virtue of its serological reactions. Thus, on carrying out the Pfeiffer reaction with L. hebdomadis and the serum of convalescents or homologous animal antisera, a positive reaction is obtained, while L. icterohæmorrhagiæ reacts negatively to these sera. Conversely, the L. hebdomadis yields a negative reaction with antisera to the infectious jaundice organism. It has been shown that the L. hebdomadis is harboured by the wild mouse—*Microtus montebelli*—and the organisms are present in the kidneys and excreted in the urine of these animals. The source and mode of infection in the human subject would appear to be analogous to that of the L. icterohæmorrhagiæ.

Dengue

Dengue is a condition presenting a fairly definite clinical picture and has been long known to have an extensive geographical distribution. Ashburn and Craig, working in the Philippines, found the blood in this disease (as in Phlebotomus fever) to be infective even after filtration through a Berkefeld filter. According to these workers, *Culex fatigans* acts as the insect vector, but it has also been supposed by others that *Aëdes* (*Stegomyia*) *calopus* may transmit the infection. Couvy has reported the isolation of a spirochæte from cases of dengue in Beirut.

The question arises in regard to these various similar conditions, to what degree they are related, and particularly in view of the claims made regarding the relationship of spirochætes of the leptospira type to seven-day fever in Japan and sandfly fever in Malta. The further question of the relationship of these conditions to infectious jaundice on account of the close similarity of the respective organisms has also been a matter of speculation. Phlebotomus fever, dengue, and seven-day

fever are probably distinct conditions. Further, dengue is specially characterised by a terminal skin eruption which is not a feature of the other conditions. It is interesting to note that the L. hebdomadis, according to the Japanese workers, is derived from mice in a manner analogous to infection with L. icterohæmorrhagiæ, while the other related infections are generally regarded as insect-borne. The same difference holds apparently for infectious jaundice and yellow fever. It seems reasonable to suppose, in the present state of our knowledge, that there is a group of pathogenic leptospiras all biologically related, though possessing different grades of pathogenicity to man and animals and serologically distinct, some of which infect wild rodents and some pass through an intermediary insect host, and that different species of this group are responsible for such conditions respectively as yellow fever, infectious jaundice, seven-day fever, and possibly phlebotomus fever, dengue, and other three-day fevers of tropical and subtropical countries.

CHAPTER XXIV

RICKETTSIA INFECTIONS

Introductory.—The term *Rickettsia* has been applied to certain definite structures found to develop in the alimentary tract of some blood-sucking insects, *e.g.* in lice after feeding on cases of typhus fever. It has also been shown that the disease is transmitted by inoculating susceptible persons with the fæces of insects containing these rickettsia bodies. Similar bodies have also been seen in the cells of the lesions in the human subject. Several other diseases have likewise been found to be transmitted by insects harbouring such bodies, namely, trench fever by lice and Rocky Mountain spotted fever by ticks, also the South African disease of cattle known as "heart-water." The rickettsia bodies are very minute diplococcal or rod-shaped structures, measuring as a rule less than 1 μ, although longer forms occur and considerable pleomorphism is shown. They are Gram-negative, but they have only a weak affinity for the ordinary bacterial stains, and in order to demonstrate them Giemsa's solution is most suitable; with this they stain of a reddish or a purple colour. The finding of the rickettsia bodies has led to much investigation regarding their occurrence in various insects and their significance. On the one hand, the specific etiological relationship of the rickettsia bodies to the respective diseases appears, in the case of lice, from the fact that insects fed upon healthy persons do not develop them and that their appearance in the gut coincides with the acquisition of infectivity by the lice. On the other hand, similar bodies have been found in various blood-sucking insects apart from the occurrence of disease in their mammalian hosts. Thus Nöller found that *Melophagus ovinus* (the sheep "ked") habitually harbours rickettsiæ, and the bed-bug (*Cimex lectularius*) is constantly infected with such bodies (Arkwright, Atkin, and Bacot). Hertig and Wolbach and others have also demonstrated their presence in a number of other insects. As regards the nature of the rickettsia bodies there is great uncertainty, although it is clear that they are definite structures distinguishable from

mitochondria, etc. That they are living objects capable of multiplication seems evident, *e.g.* in the case of typhus fever they appear in large numbers in the intestinal contents of the lice after some days following the ingestion of infected blood, and in this case also they are seen to pack the epithelial cells of the gut and to cause disturbances in function which lead to the death of the insect. Other rickettsiæ again are harmless to the insect hosts in whose tissues they appear to flourish in permanent symbiosis, being transmitted in the eggs from one generation to another. In general, attempts have failed to obtain cultures of pathogenic rickettsia bodies on artificial media, although *R. melophagi* grows on blood agar and in its behaviour in culture resembles the ordinary bacteria.

Typhus Fever

The first definite results regarding the etiology of typhus fever were obtained by Nicolle in 1909. This observer found that the blood of cases of typhus fever during the febrile period, as well as immediately before and afterwards, was infective for chimpanzees and some species of lower monkeys, in the latter especially when introduced intraperitoneally. A febrile illness which was practically identical with the disease in man (including occasionally skin eruption and sometimes fatal) was produced, and the blood of infected animals was again infective toward fresh individuals. A large number of such passages were successful. The only other animal susceptible to the infection appears to be the guinea-pig, in which, following intraperitoneal inoculation with several c.c. of infective blood, there occurs an illness after an incubation period, which is seven to twelve days as a rule, characterised by fever and loss of weight; the illness lasts for four to eleven days and is only exceptionally fatal. The virus is present in the guinea-pig during the febrile period both in the blood and also in the solid organs. In this animal likewise it can be maintained by passage and does not undergo any change in virulence. In the guinea-pig, as in the human subject, the characteristic lesion is a proliferation of the endothelial cells of the small blood vessels, which form masses in and around the lumen and lead to thrombosis and occlusion. The cellular masses are marked in the brain, but are also present in other organs and in the skin. Nicolle observed that, as in the case of man, when an experimental animal has passed through an attack of the disease it becomes immune to re-inoculation. By taking advantage of this fact

it has been shown in cross-inoculation experiments that typhus fever as met with in different parts of the world is due to the same virus; also the mild affection known in New York as Brill's disease has been proved to be an attenuated form of typhus. It has been known that children under ten years are, apparently, less susceptible to typhus than older individuals, and Nicolle made the interesting observation that when a family is attacked, young children, while apparently well, may really suffer from a slight rise of temperature. This condition is probably an abortive attack of the fever, as the blood in such cases is infective for animals. These abortive cases may play a part in the dissemination of the disease. Nicolle's results have been confirmed in America by Anderson and Goldberger and by Ricketts and Wilder. Rats show no symptoms after inoculation with typhus virus, but their blood becomes infective, as can be demonstrated by inoculating guinea-pigs. Kusama and Ségal have found that the virus is particularly associated with the blood platelets, since a suspension of these elements separated from the other constituents of the blood by centrifuging is specially infective, whereas the blood plasma and also leucocytes from peritoneal exudate do not harbour the virus. A most important fact established by Nicolle was that infection takes place through the *Pediculus humanus* (*corporis*, and probably also *capitis*). Monkeys and guinea-pigs can both be infected by lice previously fed on a human case. There is evidence that the causal organism undergoes development or multiplication in the insect host, as the louse becomes infective after the seventh day following the infected feed. During the late war there were serious outbreaks of typhus fever in Serbia, Bulgaria, and Poland, and measures founded on the view that the body louse is essential to the spread of the epidemic were always successful in controlling its spread. It is possible that the virus may be transmitted through the eggs of the insect to a second generation of lice, but this appears to be very unusual. With regard to the nature of the infective agent Nicolle found that it was destroyed by a short exposure at from 50° to 55° C. The virus is probably not filterable.

There is now a considerable amount of evidence regarding the causal relationship to typhus of *Rickettsia prowazeki*, the name given by da Rocha-Lima to certain small bodies found by himself and Prowazek in the intestine of lice taken from typhus fever patients. These are probably identical with structures described earlier by Ricketts and Wilder (who stated also that they were present in large numbers in the blood of typhus fever patients,

but this has not been confirmed), Sergent, Foley, and Vialette and others. They are non-motile bodies which are most satisfactorily demonstrated by staining with Giemsa's solution, when they appear characteristically as minute cocci, diplococci, bipolar bodies, or rods measuring 0·25×0·3–1·0 μ, usually of a reddish or lilac tint with a bluish intermediate substance; a pale capsule has also been described round the ends. But there is considerable pleomorphism, and thicker forms, threads and chains up to 4–10 μ or even 20–50 μ are also met with, the larger and thicker forms staining purple. They are Gram-negative. Growths on the ordinary culture media have not been obtained. The association of these bodies with the disease has been the subject of much investigation. They appear after an interval of about a week or longer in a proportion of lice which have been fed upon typhus patients and are kept at a suitable temperature (about 30° C.), and they are the only organisms regularly found under such circumstances. The rickettsia bodies parasitise the cells of the alimentary tract of the lice from the mid-gut downward. The infected cells become distended with the bodies, so that the nutrition of the infected insects is interfered with and they die. The rickettsia bodies are also found in enormous numbers in the fæces of infected lice. Similar bodies have been met with in lice fed upon cases of trench fever, but the latter show no tendency to invade the epithelial cells of the gut. Lice fed upon healthy persons in whom infection with typhus fever can be excluded appear never to harbour such organisms. A similar organism described by Weigl under the name of *R. rocha-limæ* as occurring in human blood, apart from obvious typhus and non-virulent for man and very feebly virulent for guinea-pigs, may be a modified form of *R. prowazeki.* The transmission of typhus infection to guinea-pigs is effected by inoculation with the gut contents of lice harbouring the rickettsia bodies, and in the absence of these bodies the guinea-pigs do not become infected. Also blood platelets of infected guinea-pigs when injected per anum into lice cause the appearance in the latter of R. prowazeki, as shown by Bacot and Ségal. The Red Cross Typhus Commission to Poland has demonstrated by special methods the rickettsia bodies in the endothelial cells of the vascular lesions of the skin, brain, and other organs from human cases, and also found them, though less frequently, in the tissues of infected guinea-pigs. In view of these facts the etiological relationship of the rickettsia bodies to typhus fever appears to be established. As regards the nature of the virus, whether bacterial or protozoal,

nothing definite is known. The excreta of infected lice remain virulent as a dry powder at room temperature for at least eleven days ; the high infectivity of such material would explain the frequency with which this disease has been contracted by investigators in the laboratory (Arkwright and Bacot). It seems probable also that the entrance of infected louse excreta into minute punctures of the skin, *e.g.* the bites of the insects and accidental scratches, constitutes the chief mode of infection in the human subject under natural conditions.

The serum of both men and animals during convalescence confers passive immunity, and also has a slight curative action, but this rapidly disappears. Nicolle and Blaizot, by immunising asses and horses with emulsions of suprarenals and spleens of guinea-pigs containing living virus, have produced an antiserum which has protective and curative effects in monkeys and man. Injections of virus killed by heat are without immunising action. Active immunisation has been effected in the human subject by injecting several small doses (1 c.c.) of serum derived from cases of typhus fever or from guinea-pigs at the height of the fever, but it is not certain whether this procedure is safe as a general measure.

In tissue cultures derived from the brain of infected guinea-pigs Wolbach and Schlesinger have found that R. prowazeki multiplies in the endothelial cells, and material from a subculture twenty-eight days after removal from the animal was shown to be infective when inoculated into a fresh guinea-pig. Various bacteria also have been obtained from the blood or tissues in typhus fever cases, most of which are clearly accidental concomitants. Plotz isolated from the blood a Gram-positive anaerobic bacillus which Olitzky and others have also cultivated from infected lice, but it is not clear that this organism bears any relationship to the disease. Bacilli of the proteus group have been cultivated from the urine of typhus fever cases which, while certainly not the causal organisms, are important because of their being agglutinated by the blood serum of patients (reaction of Weil and Felix).

The tendency for the blood serum in typhus fever to contain heterologous agglutinins was originally observed by Wilson, who found a positive Widal reaction for B. typhosus in such cases. The organisms isolated by Weil and Felix (designated Proteus–X 19) are so constantly agglutinated in high dilutions by the serum from typhus fever patients, while they are not affected by serum from other conditions, that the reaction possesses great value in diagnosis. The agglutinins for these

organisms appear in the blood early in the first week of the disease and reach their height in the second week; they may still be present many months after convalescence.

When the test is carried out by the naked-eye method (p. 125) with living suspensions of agar-slope cultures, the mixtures of serum and organisms being incubated at 37° C. for two hours, two-thirds of the cases yield a positive reaction in a dilution of 1 : 800 of the serum or greater, and less than 4 per cent. fail to cause agglutination with a dilution of 1 : 100 (Red Cross Typhus Commission). On the other hand, non-typhus sera do not agglutinate this organism in a dilution greater than 1 : 50. Thus agglutination with a 1 : 100 dilution of serum may be taken as a positive reaction (Reynolds). In order to preserve the agglutinability of suspensions of the organisms Sachs has found heating at 80° C. for an hour effective; such heated organisms agglutinate in larger flocculi than the living bacilli do, although clumping occurs more slowly than with the latter. On the other hand, heating at 56° C. destroys the agglutinability, and unheated suspensions gradually become inagglutinable on keeping. Another method of preservation consists in the addition to the suspension of 50 per cent. alcohol.

Rocky Mountain Fever

This is a typhus-like disease which has been the subject of much investigation in America. The essential pathological anatomy appears to be an inflammatory reaction of the adventitia of the vessels of the subcutaneous tissue and of the genitalia, with degenerative changes in the media, and a perivascular accumulation of large mononuclear cells. There is also thrombosis in the vessels. The disease is transmitted by a tick, *Dermacentor venustus*, which infests a variety of mammalian hosts. Monkeys, rabbits, and guinea-pigs can be infected with the blood, and also by ticks. The virus is transmitted hereditarily in the eggs of the ticks. In the guinea-pig the illness is much more severe and fatal than in typhus infection. There is fever with, in the male, swelling and hæmorrhage in the scrotum, swelling and rash in the ears, swelling and sometimes necrosis of the paws, etc. In the blood vessels in human cases and in infected guinea-pigs, especially within the large mononuclear cells and smooth muscle cells, and also in the stomach of the tick, Wolbach has found bodies 0·5 to 1 μ long, and 0·2 to 0·5 μ broad, which he has named *Dermacentroxenus rickettsi*, and which closely resemble the *R. prowazeki*. As in the case of the latter, the rickettsia bodies multiply within the endothelial cells in tissue cultures. According to Cumming the disease can be distinguished from typhus by the reaction in the guinea-pig.

The serum of animals which had recovered from the infection was found by Ricketts and Gomez to possess protective power, and Noguchi has shown that a powerful antiserum may be obtained from rabbits as a result of repeated intravenous injections of blood containing the virus. This antiserum when injected during the incubation period is effective in preventing the outbreak of the disease in guinea-pigs inoculated with many times the lethal dose. Active immunity can be developed in these animals by inoculation with fresh mixtures of living virus and antiserum.

Trench Fever

Trench fever (also known as Wolhynian fever or five-day fever) was recognised as a distinct disease only during the late war, and it has been shown to be a louse-borne infection. Though not a fatal malady, it was responsible, owing to its wide prevalence, for a serious amount of temporary disablement in the armies, while the after-effects, which occurred in a proportion of cases, were the cause of much chronic ill-health. The onset of the disease is usually sudden, and, with pyrexia, the chief symptoms are headache, giddiness, and pains in the legs, in the back, and behind the eyes, along with congestion of the conjunctivæ and sweating. There is moderate leucocytosis during the pyrexia, and the spleen is often slightly enlarged. The initial attack of marked fever, which may show fluctuations, lasts usually three to six days ; thereafter the temperature falls to normal and the symptoms subside. In many cases there occurs three or four days later a distinct relapse of shorter duration and less severity than the original attack, or there may be slight irregular pyrexia. Occasionally a regular relapsing type of fever supervenes, the temperature rising sometimes to 104° C. and falling to normal again within two days ; whilst there are intervals of about five to seven days between the attacks, during which fever and other symptoms are absent. This type of fever may occur at the beginning of the disease or may develop weeks after the primary attack. In the majority of cases complete recovery occurs comparatively soon, but patients who have had trench fever may suffer at a later stage from myalgia and rheumatic pains, irregular action of the heart, and a tendency to slight febrile attacks, and may become chronically disabled. Recovery from the disease does not lead to any marked degree of immunity.

No organism has been demonstrated either by microscopic methods or by culture in materials derived from such cases.

M'Nee and Renshaw, however, showed that the disease could be transmitted to a healthy individual by the intramuscular or intravenous injection of the blood of a patient in the acute stage. In these experiments the period of incubation varied from six to twenty-two days. Subsequently extensive investigations on this disease were carried out by a British War Office Committee working in this country and an American Research Committee in France.

It was shown that trench fever is transmitted by means of lice, both the body louse and the head louse being transmitters. The British Committee made numerous attempts to transmit the disease by the bites of lice which had previously fed on trench fever patients, but without success (the American Committee, however, observed infections produced by bites). If the excreta of such lice were collected and dried, and were used to inoculate a scarified area of the skin of healthy men, trench fever resulted in a considerable number of cases, the period of incubation being on an average about eight days. After lice were allowed to feed on a trench fever patient, a period of from five to nine days elapsed before their fæces became infective, this period suggesting a cycle of development in the louse, or indicating the time during which the organisms multiply sufficiently to produce infection. The lice remain infective for a period of at least twenty-three days after being infected, and are probably infective throughout their life. It was also found that the bodies of infected lice when crushed on the broken skin were capable of giving rise to trench fever. Even as late as fifteen months after the onset of the disease the blood during a slight febrile attack may contain the organism of the disease, as was shown by its capacity of infecting lice—a fact in accordance with the protracted character of the disease in some cases and the recrudescence of typical symptoms. It was found impossible to produce infection by the excreta of healthy lice—that is, the virus is not normally resident in the insect. Further, the infective agent is not transmitted from infected lice to their offspring. The comparative regularity with which the disease may be produced in men of various ages by infected lice shows that the proportion of naturally immune individuals is very small.

The evidence is in favour of the virus existing in the blood as an extra-corpuscular parasite. Although the virus may be present in the urine of trench fever patients, and sometimes in the sputum mixed with saliva, there is no evidence that the disease is spread otherwise than by means of lice. The virus has not been found in the fæces. The American Committee

found that on rubbing up the dried urinary sediment in saline and then passing the fluid through a Chamberland L filter, they were able to set up trench fever by injection of the filtrate. The organism may thus be, at least in one stage, a filter-passer. It is relatively resistant; it is not killed by drying, and dried excreta have been found virulent after keeping for four months; also it resists exposure to sunlight for some time. It survives an exposure to dry heat at 80° C. for twenty minutes, but when moist is killed by a temperature of 60° C. for a like period.

Töpfer described the occurrence of rickettsia bodies in lice which had fed on trench fever patients, and this has been confirmed by Arkwright, Bacot and Duncan, and others. This organism, *R. quintana,* in its microscopic appearance and staining reactions closely resembles the typhus fever rickettsia, though less pleomorphous; unlike the latter, it does not parasitise the epithelial cells of the gut in lice, but remains confined to the lumen, and the insects are not harmed by its presence. The evidence as to its etiological relationship to trench fever rests on the facts that the rickettsia bodies appear in lice five to twelve days after feeding on a trench fever patient, and that lice excreta which contain the bodies transmit the disease, whereas lice in which the possibility of having fed upon trench fever patients can be excluded do not show rickettsia and are not infective. No animal has been found susceptible to infection with trench fever virus either from blood or lice.

CHAPTER XXV

FILTER-PASSING VIRUSES: SMALLPOX, HYDROPHOBIA, MEASLES

Introduction.—While a large proportion of infective diseases have now been proved to be due to micro-organisms that can be demonstrated microscopically and, in most of such cases, cultivated artificially outside the body, there remain a certain number of prevalent diseases undoubtedly infective in nature in which the causal organism is still undefined. For many years now a category of viruses responsible for various infective diseases has been recognised, which are regarded as analogous biologically to other microbes, but are sufficiently small or plastic to pass through the pores of an earthenware or porcelain filter, *i.e.* are *filterable,* and are invisible by the ordinary microscopic methods, *i.e.* are *ultramicroscopic.* These viruses have been regarded as living organisms in virtue of their power of apparent multiplication in the tissues, as evidenced by the natural and experimental propagation of the diseases due to them. The earliest observation on this subject was made by Löffler and Frosch in 1898, who found that in foot-and-mouth disease of cattle, when the contents of one of the vesicles characteristic of the condition were diluted and passed through a Berkefeld filter, the filtrate, which was free from any micro-organisms recognisable by the microscope or by culture, was still infective like the unfiltered material. In short, the vesicle fluid appeared to contain a specific virus which was ultramicroscopic and filterable. In the following year also a filterable virus was demonstrated by Beijerinck as the causal agent of the mosaic disease of the tobacco plant. Since then many infectious diseases of man and animals have been shown to be due to such viruses, and their study has assumed great importance.

It must be remembered, however, that the ability of an organism to pass through a filter depends not only on its size but also on its plasticity, and on the fineness of the particular filter used and the pressure at which filtration is carried out. Earthenware and porcelain filters are made of various grades of

porosity, and while a filter of a certain grade may allow the passage of a particular virus, a finer filter may arrest it. Another property of the filter which may play an important part is its capacity for fixing, or, as it is termed, adsorbing, substances on its surface ; this action, which is of a physico-chemical character, depends on the nature of the material constituting the filter. In virtue of it, substances which are so finely dispersed as practically to be in solution, *e.g.* ferments, may be retained by a filter. Thus the retention of a supposed virus by a filter does not necessarily mean that it is too large to pass through the pores. It is manifestly extremely difficult, however, to control completely the porosity of a particular filter. In recent times organic membranes such as collodion, caoutchouc, etc., either as such or mechanically supported, have been employed to test filterability, but here also similar difficulty is met with. A collodion membrane, for example, as tested by the passage of various microbes, varies not only according to thickness but also according to the nature of the solution from which it has been formed. It may be noted that it has been claimed by Levaditi and Nicolau that certain viruses may pass a collodion filter which is capable of retaining the larger protein molecules, enzymes, etc. Most of the so-called filterable viruses are also ultramicroscopic. It has been estimated that the Berkefeld V filter (one of the most porous varieties) will allow the passage of particles measuring 0·2 μ in diameter which are within the range of resolution by the highest powers of the microscope, so that theoretically an organism might pass through certain grades of filters and yet be demonstrable microscopically. On the other hand, an organism of 0·1 μ or less in its longest diameter will be invisible by ordinary microscopic methods. Dark-ground illumination, which renders visible very minute bodies not demonstrable with the ordinary microscope, has not added much to our knowledge of the ultramicroscopic viruses owing to the fact that an organism of such minute dimensions, unless actively motile, cannot be distinguished from the numerous particles seen by this method in all organic material. Special photographic methods have also been used in the attempt to demonstrate these viruses as particulate structures, but they are subject to the same difficulty. Certain of the filterable viruses, however, are actually within the range of ordinary visibility by the microscope, *e.g.* the organism of bovine pleuropneumonia, which is nevertheless so small that its shape cannot be defined. Again, certain spirochætes which are demonstrable by special methods, *e.g.* the leptospiras, are apparently capable,

in virtue of their plastic structure, of passing through certain filters. Thus, when the etiology of yellow fever was first investigated, a filterable virus was demonstrated in the blood serum, and later Noguchi showed the actual virus to be a leptospira. The question has arisen, however, in regard to the filterability of certain spirochætes, whether the spirochæte itself or some supposed granular phase constitutes the filter passer. It has also been claimed that certain of the ordinary bacteria, *e.g.* the tubercle bacillus, develop a granular filterable form, but this has not been established.

The biological relationships of the ultramicroscopic viruses have been the subject of much speculation. The fact that viruses originally classified as such have proved, on further investigation, to be of spirochætal nature and that in other cases such viruses have been demonstrated as coccoid or bacilloid structures, has strengthened the view that all these viruses are microbic in nature though not necessarily belonging to a single group with uniform characters. In the case of the ultramicroscopic members of the group, their propagation has been regarded as evidence of their organismal nature. On the other hand, studies of the so-called bacteriophage have shown how propagation may possibly be simulated by a ferment-like principle (p. 28).

Attempts have been made to cultivate these various viruses artificially and to reproduce their pathogenic effects by means of such cultures, but in few cases with success. The cultivation of the virus of pleuro-pneumonia on artificial media proved successful, and it was found that this organism could be propagated through a series of subcultures without loss of virulence. In such cultures the virus was demonstrated microscopically as exceedingly minute refractile bodies, and growth was shown by the development of opalescence in fluid media and by colony formation on solid media (Nocard and Roux). The Bacterium pneumosintes of influenza was cultivated by Olitsky and Gates in the Smith-Noguchi medium (*vide* p. 569); and previous to this Flexner and Noguchi demonstrated the "globoid bodies" of poliomyelitis in the same way. On the other hand, the majority of the filter passers have not been artificially cultivated. A source of fallacy which has been detected in attempts to cultivate such viruses in certain cases is that when the medium containing a fragment of fresh tissue is incubated for a time, precipitates tend to form in the vicinity of the latter; and these may be mistaken for growths of organisms. A remarkable feature of the filterable viruses is their resistance to glycerin,

especially at low temperatures. Thus, while spore-free bacteria generally are killed by 50 per cent. glycerin, the viruses of smallpox, rabies, poliomyelitis, epidemic influenza, and herpes remain unaltered for long periods in this fluid at 4° C. This property has been utilised practically in the preservation of the vaccine virus—in calf lymph—for smallpox vaccination (*vide* p. 620). All these viruses, however, are relatively susceptible to heat and chemicals, though, as in the case of other organisms, considerable variations are met with.

The diseases of man and animals due to the filterable viruses are characterised generally by their high degree of infectiousness and rapidity of spread. Very minute doses of the virus also are capable of setting up an active infection. This has been well illustrated under experimental conditions. McCartney has shown, in the case of the herpes virus, that 0·001 c.c. of a filtrate through paper of a 10 per cent. emulsion of brain from an infected animal is sufficient to produce an experimental infection. It is also noteworthy that after recovery from an infection due to one of these viruses, an effective active immunity usually results which may be of long duration, as in the case of smallpox.

The diseases of man that have been regarded as due to filter-passing viruses are smallpox and vaccinia, chicken-pox, rabies, trachoma, measles, molluscum contagiosum, mumps, epidemic poliomyelitis, encephalitis lethargica, herpes febrilis and herpes zoster, yellow fever, phlebotomus fever and other three-day fevers of tropical and subtropical countries, dengue and the seven-day fever of the East, influenza, and common colds. The virus of typhus fever, still biologically undefined, was at one time thought to be filterable, but its filterability has not been confirmed. The common wart has also been stated to be due to a filterable principle. As mentioned above, the L. icteroides of Noguchi is now accepted as the virus of yellow fever (see p. 594), and similar spirochætes have also been demonstrated in phlebotomus fever and seven-day fever. According to Olitsky and Gates, the *Bacterium pneumosintes* constitutes the virus of influenza, and minute coccoid structures (" globoid bodies ") have been isolated from poliomyelitis and regarded as the causal agent. In smallpox, rabies, etc., characteristic cell inclusions have been observed in the affected tissues, which were originally thought to be parasitic organisms of protozoal nature.

In addition, a number of prevalent animal diseases are due to similar viruses—foot-and-mouth disease, bovine pleuro-

pneumonia, sheep- and cow-pox, hog cholera, cattle plague, African horse sickness, fowl pest, pigeon-pox, and various others. A filterable causal agent was also demonstrated by Rous in 1911 in cases of fowl sarcoma. Evidence has recently been brought forward by Gye and Barnard that carcinoma is caused by a filter-passing virus acting in association with an accessory chemical factor. The latter is in some way the product of the particular cells which take on the neoplastic character, while the virus appears to be common to various growths. Both, however, are essential for the starting of a tumour, which may be regarded as the result of the action of the virus on a cell whose biological characters have been in some way altered by conditions such as chronic irritation, etc. It is as yet too early to estimate the true significance of their results.

Levaditi has pointed out that a group of filter-passing viruses, namely, those of rabies, vaccinia, poliomyelitis, encephalitis, and herpes, resemble one another, not only in their general characters, but also in possessing the common feature of having an affinity for the structures derived from the epiblast—skin, cornea, etc., on the one hand, and nervous system on the other. Such affinity is shown in the case of any particular virus by its flourishing in such structures and producing lesions. He has applied the general term *ectodermoses* to the resulting lesions, and regards these as being of two main types, namely, the *neurotropic* and the *dermotropic*, according to the affinity of the virus. He has arranged the viruses according to their affinities, and regards the poliomyelitis virus as the most strictly neurotropic, since not even infection occurs by the skin, while this takes place in the case of rabies though no lesion results. The vaccinia virus is at the other extreme of the series, being specially dermotropic, while the viruses of encephalitis and herpes occupy an intermediate position, producing lesions both in the skin and central nervous system, as is described below. It may be mentioned in this connection that it has been shown by Levaditi and others that the vaccinia virus can be adapted to growth in the cerebral tissue and thereafter maintained indefinitely in series, and that it then possesses a fixed virulence, as in the case of the virus of rabies. Such views are suggestive, but it is not yet possible to estimate their significance.

General Principles Applicable in the Demonstration of a Filterable Virus.—Rigid precautions must be exercised in attempting to demonstrate such viruses in order to obviate fallacious results. Every filter employed must have been proved to arrest the ordinary bacteria. The filtration should be carried out within the minimum time, but the positive or negative pressure used in

filtration should be as low as possible—50 mm. of mercury, if possible, and never exceeding 500 mm. Fluids rich in protein, *e.g.* blood serum, must be diluted 10- to 50-fold to facilitate their filtration and to avoid clogging of the filter. The demonstration of the virus will depend on the experimental production of the characteristic disease in susceptible animals (or, in some cases, human volunteers) by inoculation of the filtrate obtained from material likely to contain the virus (*e.g.* blood serum, nasal and throat secretions, tissue emulsions, inflammatory exudates). A convenient method of controlling the permeability of the filter is to add an emulsion of B. prodigiosus [1] to the material before filtration and test the filtrate for sterility by the usual culture methods To establish the occurrence of propagation on the part of the virus, passage through a series of susceptible animals is carried out after the first experimental results are obtained. In any preliminary experiments, one of the coarser filters is used, *e g.* a Berkefeld V or Chamberland F. In later tests, finer filters may be employed to estimate the degree of filterability of the virus.

Smallpox and Vaccination

Smallpox is a disease to which much study has been devoted owing, on the one hand, to the havoc which it formerly wrought in Europe, and, on the other hand, to the controversies which have arisen in connection with the active immunisation against it introduced by Jenner. Though there is no doubt that a *contagium vivum* exists, the causal organism has not been identified or cultivated.

Jennerian Vaccination.—Up to Jenner's time the only means adopted to mitigate the disease had been by inoculation (by scarification) of virus taken from a smallpox pustule, especially from a mild case ; by this means a mild form of the disease was often originated. It had previously been known that one attack of the disease protected against future infection, and that the mild attack produced by inoculation also had this effect. This inoculation method had long been practised in various parts of the world, and had considerable popularity all over Europe during the eighteenth century. Its disadvantage was that the resulting disease, though mild, was still infectious, and thus might be the starting-point of a virulent form among unprotected persons. Jenner's discovery was published when inoculation was still considerably practised. It was founded on the popular belief that those who had contracted cowpox from an affected animal were insusceptible to subsequent infection from smallpox. In the horse there occurs a disease known as horsepox, especially tending to arise in wet, cold springs, which

[1] A small saprophytic bacillus which in culture at room temperature produces a pink pigment, thus rendering the colonies easily recognisable.

consists in an inflammatory condition with vesicle formation about the hocks, giving rise to ulceration. Jenner believed that the matter from these ulcers, when transferred by the hands of those who dressed the sores to the teats of cows subsequently milked by them, gave rise to cowpox in the latter. This disease was thus, in his opinion, identical with horsepox, and it had its origin in epidemics of the latter. Cowpox manifests itself as a papular eruption on the teats; the papules become pustules; their contents dry up to form scabs, or more or less deep ulcers occur at their sites. From such a lesion the hands of the milkers may become infected through abrasions, and a similar local eruption occurs, with general symptoms in the form of slight fever, malaise, and loss of appetite. It is this illness which, according to Jenner, gave rise to immunity from smallpox infection. He showed experimentally that persons who had suffered from such attacks did not react to inoculation with smallpox; and further, that persons to whom he communicated cowpox artificially were similarly immune. The results of Jenner's observations and experiments were published in 1798 under the title, *An Inquiry into the Causes and Effects of the Variola Vaccinæ*. Though from the first Jennerian vaccination had many opponents, it gradually gained the confidence of the unprejudiced, and became extensively practised all over the world, as it is at the present day.

The so-called vaccine lymph which contains the protecting agent is the serous exudate of the cowpox vesicle. When such lymph is used for inoculating calf from calf by *passage* a continuous supply of a product of very constant potency is obtained; this is the usual source of the lymph used for human vaccination. By its use immunity against smallpox is conferred on the vaccinated individual for a considerable period. It has been objected that some of the lymph which has been used has been derived from calves inoculated, not with cowpox, but with human smallpox. It is possible that this may have occurred in some of the strains in use shortly after the publication of Jenner's discovery, but most of the modern strains have been derived originally from cowpox. The most striking evidence in favour of vaccination is derived from its effects among the staffs of smallpox hospitals; for here, in numerous instances, it is only the unvaccinated individuals who have contracted the disease. While vaccination is undoubtedly efficacious in protecting against smallpox, Jenner over-estimated the duration of the immunity. It has been noted in smallpox epidemics that whereas young unprotected subjects readily contract the disease

those vaccinated as infants escape more or less till after the thirteenth to the fifteenth years. Revaccination is therefore necessary if immunity is to continue; and where this is done in any population, smallpox becomes a rare disease, and the mortality is practically nil. The whole question of the efficacy of vaccination was investigated in this country in 1896 by a Royal Commission, whose general conclusions were as follows. Vaccination diminishes the liability to attack by smallpox, and when the latter does occur, the disease is milder and less fatal. Protection against attack is greatest during nine or ten years after vaccination. It is still efficacious for a further period of five years, and possibly never wholly ceases. The power of vaccination to modify an attack outlasts its power wholly to ward it off. Revaccination restores protection, but this operation must be from time to time repeated. Vaccination is beneficial according to the thoroughness with which it is performed.

The Relationship of Smallpox (Variola) to Cowpox (Vaccinia).—This is a question regarding which great controversy has taken place; a subsidiary point has been the inter-relationships within the group of animal diseases which includes cowpox, horsepox, sheeppox, etc. With reference to smallpox and cowpox the problem has been—are the viruses identical or not? There is no doubt that cowpox can be communicated to man, in whom it produces the vesicle limited to the point of inoculation, and the slight general symptoms which vaccination with calf lymph has made familiar. On the other hand, it never reproduces in man a general eruption, and the local eruption is only infectious when matter from it is introduced into an abrasion. (In the parallel condition in the guinea-pig, however, Camus has produced a general eruption by the intravenous injection of calf lymph.) Moreover, the loss of infectiveness by transmission through the body of a relatively insusceptible animal is a condition which is familiar in other diseases, and the uniformity of the type of the affection resulting from vaccination with calf lymph finds a parallel in hydrophobia, where, after passage through a series of monkeys, a virus of attenuated but constant virulence can be obtained. Monkeys are susceptible to both vaccinia and variola. On inoculating the shaved skin by scarification a papulo-vesicular lesion results in each case, and the lesion can be indefinitely maintained in series. Further, monkeys thus inoculated with lymph from vaccinia are rendered immune against variola, and the converse holds; though Gordon found that the degree of protection produced by vaccinia against variola was rather greater than that produced by

variola against vaccinia. In the rabbit, on the other hand, similar inoculation with vaccinia gives rise to the typical lesion, whereas inoculation with variola is practically without effect. The calf likewise is comparatively refractory to variola, and the typical lesions are not produced at first; in fact, it has been held by some that it is not possible to variolise the calf in this way. Sometimes, however, slight reaction occurs at the site of inoculation, and if inoculation is made from the lesion to another calf, and then continued in series, the lesions may gradually become more marked and ultimately assume the characters of those produced by vaccinia. Thereafter, the altered virus may be indefinitely transmitted. This result has been repeatedly obtained, and the animals thus treated are immune against natural vaccinia. Not only so, but on using for human vaccination the lymph from such variolated calves, results indistinguishable from those produced by vaccine lymph are obtained, and the transitory illness which follows, unlike that produced in man by inoculation with smallpox lymph, is no longer infectious. In fact, many of the strains of lymph in use in Germany at present have been derived thus from the variolation of calves. Thus all the facts available go to show not only that vaccinia confers immunity against variola, but that variola confers immunity against vaccinia. In the absence of proof based on the isolation of identical organisms from the two conditions, we are at present justified in considering that vaccinia and variola are due to the same virus, and that the differences between them result from the relative susceptibilities of the two species of animals in which they occur naturally. Further, the results of investigation of the antibodies in the blood of highly immunised animals are in conformity with this (*vide infra*).

With regard to the relation of cowpox to horsepox, it is probable that they are the same disease. Both are characterised by localised eruptions, and both appear to be ordinarily transmitted by human agency. Some outbreaks of cowpox have originated from the horse, but in other cases such a source has not been traced. Sheeppox, which occurs chiefly in south-eastern Europe, is an interesting affection, as it presents certain analogies to human smallpox. It is a highly infectious disease, characterised by a generalised eruption, and is believed to be acquired by inhalation. Inoculation of the sheep with lymph from a vesicle usually produces only a local lesion (though sometimes there is a slight general eruption), and thereafter the animal is immune. Inoculation of herds is carried out as a preventive measure and is effective, but the animals thus treated are still infectious to fresh animals. Vaccinia lymph does not protect the sheep against sheeppox, nor does sheeppox lymph protect the ox against vaccinia. Goatpox presents simi-

larities to sheeppox, but goats do not become infected during outbreaks of sheeppox, and lymph from the latter is practically without effect on the goat. Swinepox, a disease also with disseminated eruption, is rare; infection is said to come from vaccinia. It has been generally concluded from these and other observations that there are several varieties of viruses, but that the two main types are those of cowpox and sheeppox. Gins, however, by means of passage through rabbits, succeeded in transforming sheeppox and goatpox, as well as swinepox, into cowpox; and accordingly the different viruses are apparently to be regarded as modifications of the same.

Alastrim.—During recent years much attention has been directed to a variola-like disease which is characterised by the relative absence of constitutional disturbances and by its non-fatal character, whilst the skin lesions may be severe, sometimes of a confluent type. It is highly infectious, and epidemics have occurred practically all over the world. Various names have been applied, such as para-variola, varioloid, mild smallpox, and alastrim; the last term has now come into general use. There has been much controversy with regard to its relation to variola, but we can refer only to the experimental work. The results of inoculation on animals are closely similar to those in the case of variola. In monkeys the lesions are the same with the two viruses, though Gordon found that those of the alastrim virus were rather less severe. As in the case of variola, rabbits and calves are resistant to the alastrim virus or only slight lesions occur, but Blaxall found that on passage through the calf the alastrim virus became so modified that it produced the typical lesions of vaccinia. Immunity studies have brought out no important differences. Vaccinia protects against alastrim as it does against variola, though differences in degree may be noted, and Gordon has found that the antibodies in anti-vaccinia serum react also with the alastrim virus. All the evidence at present available goes to show that the alastrim virus represents a modified form or variant of the variola virus, the modification consisting mainly in the loss of the toxic and lethal properties towards the human subject, while the pathogenic action in animal tests and the antigenic properties have been little altered.

Nature of Virus.—It has been conclusively shown that the virus of variola and of vaccinia is not a bacterium. In the lymph of skin lesions bacteria are, of course, present, but these can be removed or killed without the infective properties of the lymph being affected. Noguchi, for example, obtained this result by passage through the testes of rabbits. The filtration

of vaccine lymph through a Berkefeld filter has given rather divergent results. Sometimes the filtrate is infective, sometimes not. The result apparently depends upon the amount of adsorption which occurs in the pores of the filter, as it has been shown that substances like kaolin, animal charcoal, phosphate of calcium, etc., absorb the vaccinia virus and others of similar nature. Levaditi and Nikolau have shown that the virus can pass through a film of collodion rather more easily than the viruses of rabies and encephalitis, and they consider that this indicates that in magnitude the virus probably corresponds to the larger protein molecules. On the other hand, Gordon has found that the virus is affected by gravity and is largely thrown down on centrifuging, and concludes that it is of particulate character. Very minute bodies have been observed in infective lymph and also in lesions, by dark-ground illumination and other methods, but it is doubtful whether any of these represent the actual virus. It is now generally accepted that the virus belongs to the filter-passing group, but it is not possible to say what its actual size is, and there is also the possibility that what we call the " virus " may sometimes be composed of aggregates. The virus has not been cultivated outside the body.

The virus is comparatively thermolabile, its pathogenic properties being practically destroyed on exposure for an hour at 55° C. though it has still some antigenic power (Gordon). It is readily destroyed by various antiseptics, but in this respect its behaviour corresponds to other filterable viruses rather than to bacteria. For example, it persists practically unchanged in 50 per cent. glycerin and in 10 per cent. ether, its resistance to the former being taken advantage of in the preparation of vaccine lymph for preventive inoculation. Gordon has found that the virus both in vaccine lymph and in variolous material is very susceptible to the action of potassium permanganate, being readily destroyed by a solution of 1 : 10,000. In fact, this substance is virulicidal in higher dilutions than is carbolic acid or even corrosive sublimate.

Certain peculiar structures are to be found within the epithelial cells of the smallpox and vaccinia lesions both in the natural disease and in that experimentally produced, *e.g.* in the cornea of the rabbit, which is a suitable site for study. They have been specially investigated by Guarnieri, Councilman, Ewing, Prowazek, and others, but their nature is still a matter of dispute. They are usually globoid, measuring about 1–4 μ in diameter, though they may be smaller; sickle-shaped and other forms are met with. They are contained within the epithelial cells, often lying near the

nuclei, and stain with ordinary nuclear dyes. Supposed dividing and budding forms have been described, and even a cycle of development. There is no doubt that these structures, or " vaccine bodies " as they are frequently called, are a feature of the lesions, but there is no evidence that they are protozoal, as some have supposed. In all probability they represent a degenerative or possibly reactive change due to the presence of the virus in the cells. It has been supposed that the bodies actually form around minute chromatic granules representing the virus, so constituting a mantle, as it were. Prowazek considers that the granules observed by him are living organisms, and, in view of their property of leading to such formations around them, has applied the term *chlamydozoa* to them. This view cannot be regarded as established. The formations described are probably to be regarded as belonging to the same class as the bodies met with in the lesions of trachoma, hydrophobia (p. 626) and other diseases.

Antibodies.—Various facts have been mentioned above with regard to protection afforded by cutaneous inoculation, but it has been shown that immunity can also be produced in animals by introducing the virus of vaccinia by various routes—subcutaneous, intravenous, etc. Gordon has studied further the passage of the virus through intact surfaces, and has found that the nasal mucosa is the most permeable, a catarrhal condition resulting which is attended by immunity to cutaneous inoculation. This observation is manifestly of importance in relation to the possibility that infection in smallpox takes place through the respiratory passages. In animals immunised by the above methods, antibodies appear in the blood of a nature corresponding to those recognised in bacterial infection—agglutinating, complement-fixing, and virulicidal bodies. The presence of the last variety is shown by the fact that when the virus is exposed for some time to the antiserum it is bereft of its infective properties. Gordon has recently made an extensive study of the antibodies in anti-vaccinia serum, and has found that they combine with and give agglutinating and complement-fixing reactions with the viruses of variola and alastrim as well as with that of vaccinia. It may also be added that corresponding observations have been made on the sera of patients who have recovered from smallpox, and here also antibodies have been demonstrated. It has been found that such sera give complement-fixation not only with material from the lesions of variola but also with the virus of vaccinia. Further, Schneider showed that the serum of variola convalescents had a distinct virulicidal action on vaccinia virus. Phenomena of supersensitiveness have been observed on re-inoculating with virus, and here also the action of the two types is reciprocal. All these facts support

the view already expressed that vaccinia virus is simply the virus of variola modified in certain of its properties by passage through the bovine species. We may state in conclusion that the virus obtained from the lesions of varicella (chicken-pox) gives no reactions with an anti-vaccinia serum, and that no cross immunity reactions between the two viruses have been observed. It is generally accepted that varicella is quite a distinct disease.

For the differentiation of variola and varicella great value attaches to the results produced on inoculation of the rabbit's cornea with the contents of the skin lesions (Paul). The cornea is lightly scarified and the material (which if previously dry is rubbed up in a drop of saline) introduced into it. After thirty-six to forty-eight hours if the material has been derived from a case of variola, small, clear, bubble-like elevations have developed on the cornea, which are best seen as opaque white spots when the eye is enucleated (the cornea being then cleansed of any adherent blood or secretions) and placed for a short time —about a minute—in a mixture of 2 parts saturated watery solution of corrosive sublimate with 1 part of absolute alcohol. Vaccinia causes an appearance which is similar, but more marked; but varicella and other infections produce nothing likely to be mistaken for it. The value of this procedure has been verified by Ungermann and Zuelzer and others.

Hydrophobia or Rabies

Introductory.—Hydrophobia or rabies is an infectious disease which in nature occurs epidemically chiefly among the carnivora, especially in the dog and the wolf. Infection is carried by the bite of a rabid animal or by a wound or abrasion being licked by such. The disease can be transferred to other species, and when once started can be spread from individual to individual by the same paths of infection. Thus it occurs epidemically from time to time in cattle, sheep, pigs, horses, and deer, and can be communicated to man. Cases of infection from man to man by bite are recorded, but the saliva in man does not appear to be so infectious as in dogs. It is to be noted that the virus is apparently extremely potent, as cases of infection taking place through an unabraded mucous membrane by the licking of a rabid animal are on record, and the experimental application of the virus to such surfaces as the mucous membrane of the nose or the conjunctiva is often followed by infection.

In Western Europe the disease is most frequently observed in the dog; but in Eastern Europe, especially in Russia,

epidemics among wolves constitute a serious danger both to other animals and to man. Two varieties of rabies are recognised—(1) rabies proper, or furious rabies (*la rage vraie, la rage furieuse; die rasende Wuth*); and (2) dumb madness or paralytic rabies (*la rage mue; die stille Wuth*). The disease, however, is essentially the same in both cases. In the dog the furious form is the more common. After a period of incubation of from three to six weeks, the first symptom noticed is a change in the animal's aspect; it becomes restless, it snaps at anything which it touches, and tears up and swallows unwonted objects; it has a peculiar, high-toned bark. Spasms of the throat muscles appear, especially in swallowing, and there is abundant secretion of saliva; its supposed special fear of water is, however, only part of the fear it has for swallowing generally. Gradually convulsions, paralysis, and coma come on; and death supervenes usually about five days after the appearance of symptoms. In the paralytic form, the early symptoms are the same, but paralysis appears sooner. The lower jaw of the animal drops, from implication of the elevator muscles, all the muscles of the body become more or less weakened, and death ensues without any very marked irritative symptoms.

In man the incubation period after infection varies from fifteen days to seven or eight months, or even longer, but is usually about forty days. When symptoms of rabies are about to appear, certain prodromata, such as pains in the wound and along the nerves of the limb in which the wound has been received, may be observed. To this succeeds a stage of nervous irritability, during which all the reflexes are augmented—the victim starting at the slightest sound, for example. There are spasms, especially of the muscles of deglutition and respiration, and cortical excitement evidenced by delirium may occur. On this follows a period in which all the reflexes are diminished, weakness and paralysis are observed, convulsions occur, and finally coma and death supervene. The duration of the acute illness is usually from four to eight days, and death invariably results. The existence of paralytic rabies in man has been denied by some, but it undoubtedly occurs. This is usually manifested at first by paralysis of the limb in which the infection has been received, and of the neighbouring parts, and then the occurrence of widespread and progressive paralysis is the outstanding feature. In man there also occur cases where the cerebellum and also the sympathetic system seem to be specially affected.

The Pathology of Hydrophobia.—In hydrophobia as in

tetanus, to which it bears more than a superficial resemblance, the appearances discoverable by an ordinary examination of the nervous system, to which all symptoms are naturally referred, are comparatively unimportant. On naked-eye examination, congestion, and, it may be, minute hæmorrhages, especially in the medulla, are the only features noticeable. Microscopically, lymphocytic exudation into the perivascular lymphatic spaces in the nerve centres has been observed, and in the nerve cells various degenerations have been described. Round the nerve cells in the grey matter of the medulla and cord, Babès described accumulations of newly formed cells, and Van Gehuchten observed a phagocytosis of the nerve cells in the posterior root ganglia and also in the sympathetic ganglia. The most important feature, however, is the presence of the structures known as Negri bodies in the nerve cells (p. 626). In the white matter, especially in the posterior columns, swelling of the axis cylinders and breaking up of the myelin sheaths have been noted, and similar changes may occur also in the spinal nerves, especially of the part of the body through which infection has come. The changes in the other parts of the body are unimportant.

Pasteur's first contribution to the pathology of rabies was to show that the most certain method of infection was by inserting the infective matter beneath the dura mater. He found that in the case of any animal or man dead of the disease, injection by this method of emulsions of any part of the central nervous system, of the cerebro-spinal fluid, or of the saliva, invariably gave rise to rabies, and also that the natural period of incubation was shortened. Further, the identity of the furious and paralytic forms was proved, as sometimes the one, sometimes the other, was produced, no matter which form had been present in the original case. Inoculation into the anterior chamber of the eye is nearly as efficacious as subdural infection. Infection with the blood or solid organs of rabid animals does not reproduce the disease, though there is evidence that the poison exists in such glands as the pancreas and mamma. *Subcutaneous* infection with part of the nervous system of an animal dead of rabies may or may not give rise to the disease.

In consequence of the introduction of such reliable inoculation methods, further information has been acquired regarding the spread and distribution of the virus in the body. Gaining entrance by the infected wound, it early manifests its affinity for the nervous tissues. It reaches the central nervous system chiefly by spreading up the peripheral nerves. This can be

shown by inoculating an animal subcutaneously in one of its limbs with virulent material. If now the animal be killed before symptoms have manifested themselves, rabies can be produced by subdural inoculation from the nerves of the limb which was infected. Further, rabies can often be produced from such a case by subdural infection with the part of the spinal cord into which these nerves pass, while the other parts of the animal's nervous system do not give rise to the disease. This explains how the initial symptoms of the disease (pains along nerves, paralysis, etc.) so often appear in the affected part of the body, and it probably also explains the fact that bites in such richly nervous parts as the face and head are much more likely to be followed by hydrophobia than bites in other parts of the body. Again, injection into a peripheral nerve, such as the sciatic, is almost as certain a method of infection as injection into the subdural space, and gives rise to the same type of symptoms as injection into the corresponding limb. Intravenous injection of the virus, on the other hand, differs from the other modes of infection in that it more frequently gives rise to paralytic rabies. This fact Pasteur explained by supposing that the whole of the nervous system in such a case becomes simultaneously affected. In certain animals the virus seems to have an elective affinity for the salivary glands, as well as for the nervous system. Roux and Nocard found that the saliva of the dog became virulent three days before the first appearance of symptoms of the disease.

The Virus of Hydrophobia.—The only definite information we possess regarding the causal organism of rabies is that in one stage of its life-history at any rate it must be extremely small, as it can pass through the coarser Berkefeld filters and also sometimes through the coarser Chamberland filters. This is shown by the fact that if an emulsion of any infective material (*e.g.* the brain) be thus filtered, the filtrate is also infective. Evidence that it is the organism itself which passes through is found in the fact that when an animal dies on injection with the filtrate, a small portion of its central nervous system will originate the disease in a fresh animal. Like the virus of variola, it can also pass through a collodion membrane (Levaditi and Nicolau). It is thus placed among the filterable viruses. A toxin may, however, be concerned in the production of the pathogenic effects. Remlinger found that death with paralytic symptoms followed the injection of filtered virus, but that the nervous system of the dead animals sometimes did not reproduce rabies. He explained this occurrence by supposing that

under such circumstances the filtrate contained a toxin but not the actual infective agent. The resistance of the virus to external agents varies. Thus a nervous system containing it is virulent till destroyed by putrefaction; it can resist the prolonged application of a temperature of from −10° to −20° C., but, on the other hand, it is rendered non-virulent by one hour's exposure at 50° C. Again, its potency probably varies in nature according to the source. Thus, while the death-rate among persons bitten by mad dogs is about 16 per cent., the corresponding death-rate after the bites of wolves is 80 per cent. Here, however, it must be kept in view that, as the wolf is naturally the more savage animal, the number and extent of the bites, *i.e.* the number of channels of entrance of the virus into the body and the total dose, are greater than in the case of persons bitten by dogs. As we shall see, alterations in the potency of the virus can certainly be effected by artificial means, such as drying, heating, and applying chemical agents. Various attempts have been made to obtain cultures from the hydrophobic virus, but convincing results have not been obtained.

Negri bodies.—In 1903, Negri described certain bodies as occurring in the nervous system in animals dying of rabies to which much attention has since been devoted, and regarding the significance of which opinion is still divided. Negri's observations have been generally confirmed, and it is probable that the occurrence of the bodies is specific to the disease.

The Negri bodies (Plate IV., Fig. 16)[1] can be readily found in the affected nervous system by making smear preparations or sections, as described below (p. 631). They vary much in size, measuring 0·5–25 μ; in the dog as a rule they are 4–10 μ. They are round, oval, or somewhat angular in outline. They are found in the protoplasm of the nerve cells and of their processes. When examined in unstained preparations, they are seen to have a sharply defined outline, and some of the features of the internal structure presently to be described can be noted. For the finer differentiation of the internal structure, Negri employed Giemsa's stain. With this stain and under high magnification the groundwork of the body is a pale blue; in it there appear certain round or oval formations, single or multiple, of varying size, stained pink and of homogeneous appearance (*grosse Innenformationen*). In addition, both inside these larger formations and in the general substance of the body are smaller red or violet-red granules, occurring singly

[1] For the material from which this preparation was made we are indebted to Lt.-Col. W. F. Harvey, I.M.S.

or in clumps (*kleine Innenformationen*). Though the description given applies to Giemsa preparations, any eosin-methylene-blue stain is suitable for their demonstration. Their exact staining reactions vary with the method used, but they tend to be eosinophile.

The Negri bodies have been found in practically 98 per cent. of cases of street rabies in dogs examined by many observers in different parts of the world. They are also found in natural rabies in other animals, and are usually present in human cases. Numerous control observations on other toxic conditions of the nervous system, especially where these are characterised by spasms, have been made, and the consensus of opinion is that the presence of Negri bodies is a specific appearance in nerve cells and justifies a positive diagnosis of rabies. The bodies occur in all parts of the nervous system, but are specially abundant in the cells of the cornu Ammonis (hippocampus major) and in the Purkinje cells of the cerebellum. It is in the former situation that they are generally looked for. They are apparently not so readily found, and at any rate the larger forms may be altogether absent, in animals dying from inoculation with the exalted fixed virus. Hitherto they have not been certainly found in the salivary glands or saliva of a rabid animal.

While there is a general tendency to recognise the Negri bodies as being specific to rabies, great difference of opinion exists as to their true nature. Negri himself looks upon them as protozoa, and the organism has been named by Calkins *Neuroryctes hydrophobiæ*. This view is based upon their relatively constant and peculiar structure which, such authorities as Golgi state, does not correspond to any cellular degeneration. And the fact that the nerve cells in which they are present may show no evidence of degeneration, is also noteworthy. Against their protozoal nature has been urged their absence from the virulent brains of some animals dying from fixed virus, their non-discovery in the infected saliva, and the fact that the virus can pass through a coarse filter. These objections have been met with the argument that the smaller internal formations may be the infective agent in its essential form, and a modification of this view is that the Negri body is the result of a cellular reaction against an invasion by these ultimate forms. This latter view appears to us to have most in support of it, but the whole question is still matter of controversy.

The Prophylactic Treatment of Hydrophobia.—Until the publication of Pasteur's researches in 1885, the only means

adopted to prevent the development of hydrophobia in a person bitten by a rabid animal had consisted in the cauterisation of the wound. Such a procedure was undoubtedly not without effect. It has been shown that cauterisation within five minutes of the infliction of a rabic wound prevents the disease from developing, and that if done within half an hour it saves a proportion of the cases. After this time, cauterisation only lengthens the period of incubation ; but, as we shall see presently, this is an extremely important effect.

The work of Pasteur, however, revolutionised the whole treatment of wounds inflicted by hydrophobic animals. Pasteur started with the idea that, since the period of incubation in the case of animals infected subdurally from the nervous systems of mad dogs is constant in the dog, the virus has been from time immemorial of constant strength. Such a virus, of what might be called natural strength, is usually referred to in his works as the virus of *la rage des rues*,[1] in the writings of German authors as the virus of *die Strasswuth*. Pasteur found on inoculating a monkey subdurally with such a virus, and then inoculating a second monkey from the first, and so on with a series of monkeys, that it gradually lost its virulence, as evidenced by lengthened periods of incubation on subdural inoculation of dogs, until it wholly lost the power of producing rabies in dogs, when introduced subcutaneously. When this point had been attained, its virulence was not diminished by further passage through the monkey. On the other hand, if the virus of *la rage des rues* were similarly passed through a series of rabbits or guinea-pigs, its virulence was increased till a constant strength (the *virus fixe*) was attained—constancy of strength being indicated by the unvarying occurrence of paresis on the sixth day. Pasteur had thus at command three varieties of virus—(*a*) that of natural strength, (*b*) that which had been attenuated, and (*c*) that which had been exalted. He further found that, commencing with the subcutaneous injection of a weak virus, and following this up with the injection of the stronger varieties, he could ultimately, in a very short time, immunise dogs against subdural infection with a virus which, under ordinary conditions, would certainly have caused a fatal result. He also elaborated a method by which the exalted virus contained in the spinal cords

[1] While Pasteur's original statements regarding the constancy of the virulence of the street-virus were probably accurate for the street dogs of Paris, it has been found that if the general virulence of virus derived from animals in nature be studied, considerable variation occurs. It is now usual to apply the term street-virus to any virus derived from an animal becoming rabid under natural conditions of infection.

of rabbits could be attenuated. This was done by drying the cords in air over caustic potash (to absorb the moisture), the diminution of virulence being proportionate to the length of time during which the cords were kept. Accordingly, by taking a series of such spinal cords kept for various periods of time, he was supplied with a series of vaccines of different strengths. Pasteur at once applied himself to find whether the comparatively long period of incubation in man could not be taken advantage of to " vaccinate " him against the disease before its gravest manifestation took place.

In the first case treated the first injection was emulsion of rabbit's cord dried for fourteen days, and this was followed by injections of cord less attenuated. Treatment was continued over nine days, the last injection being one of emulsion of cord dried for only one day, *i.e.* containing approximately *virus fixe.* The patient never manifested the slightest symptom of hydrophobia. Other similarly favourable results followed ; and this prophylactic treatment of the disease quickly gained the confidence of the scientific world, which it still retains.

An important modification in the method which further experience led Pasteur to make, was in the treatment of serious cases, such as multiple bites from wolves, extensive bites about the head, especially in children, cases which come under treatment at a late period of the incubation stage, and cases where the wounds have not cicatrised. In such cases the stages of the treatment are condensed. Thus on the first day, say at 11 a.m and 4 p m. and 9 p.m., cords of 12, 10, and 8 days respectively are used ; on the second day, cords of 6, 4, and 2 days ; on the third day, cords of 1 day ; on the fourth day, cords of 8, 6, and 4 days ; on the fifth, cords of 3 and 2 days ; on the sixth, cords of 1 day ; and so on for ten days. In each case the average dose is about 2 c.c. of the emulsion.

The method of prophylactic treatment will thus be seen to be simply one of producing active immunity by successive injections of virus of gradually increasing potency, and the possibility of this being carried out *after* the infective virus has been introduced into the body is due to the long period of natural incubation. Details of the method as regards dosage and virulence of material used vary in different Pasteur institutes. The most important modifications which have within recent times taken place are (1) the substitution by Högyes of increasing concentrations of a fairly fresh virulent rabbit's cord for emulsions of cords subjected to decreasing periods of drying, and (2) the use of material prepared from the brain and medulla of rabbits infected with fixed virus—the virus having been killed and

preserved with carbolic acid (Semple). Equally good results apparently are obtained by these methods. It is stated that in cases treated by Högyes' method, certain symptoms sometimes following the ordinary treatment, the gravest of which may be the occurrence of temporary paralysis, are not so frequently observed. This, according to Harvey and M'Kendrick, who have studied the subject very fully, may be due to the fact that a smaller amount of nerve tissue is injected under the Högyes system. Semple's method is now widely used.

The success of the treatment has been very marked. The statistics of the cases treated in Paris are published annually in the *Annales de l'Institut Pasteur*. The ordinary mortality is probably 16 per cent. of all persons bitten. During the ten years 1886–95, 17,337 cases were treated, with a mortality of 0·48 per cent., and recent statistics show even more favourable results. It has been alleged that many people are treated who have been bitten by dogs that were not mad. This, however, is not more true of the cases treated by Pasteur's method than it was of those on which the ordinary mortality of 16 per cent. was based, and care is taken in making up the statistics to distinguish the cases into three classes. Class A includes only persons bitten by dogs proved to have had rabies, by inoculation in healthy animals of parts of the central nervous system of the diseased animal. Class B includes those bitten by dogs that a competent veterinary surgeon has pronounced to be mad. Class C includes all other cases. During 1895, 122 cases belonging to Class A were treated, with no deaths; 940 belonging to Class B, with two deaths; and 449 belonging to Class C, with no deaths. Besides the Institute in Paris, similar institutions have been established in Italy, in Russia, and in India, as well as in other parts of the world; and in these similar success has been experienced. It may be now taken as established, that a very grave responsibility rests on those concerned if a person bitten by a mad animal is not subjected to the Pasteur treatment. Sometimes during or after treatment there appear slight paralytic symptoms with obstinate constipation and it may be retention of urine, but these usually pass off within a few weeks and leave behind no ill-effects.

Antirabic Serum.—In the early part of the nineteenth century an Italian physician, Valli, showed that immunity against rabies could be conferred by administering through the stomach progressively increasing doses of hydrophobic virus. Following up this observation, Tizzoni and Centanni have attenuated rabic virus by submitting it to peptic digestion, and have immunised animals by injecting gradually increasing strengths of such virus. This method is usually referred to as the Italian method of immunisation. The latter workers showed from this that the serum of animals thus immunised could give rise to passive immunity in other animals; and further, that if injected into animals from seven to fourteen days after infection with the virus, it prevented the latter from producing its fatal effects, even when symptoms had begun to manifest themselves. They further succeeded in

producing in the sheep and the dog an immunity equal to from 1 : 25,000 to 1 : 50,000 (*vide* p. 189), and they recommended the use, in severe cases, of the serum of such animals in addition to the treatment of the patient by the Pasteur method. Marie obtained a similar serum by subcutaneous injection of the sheep with *virus fixe*. The use of this serum to supplement the ordinary Pasteur treatment has been found beneficial in severe human infections, and in ordinary cases it enables the prophylactic injections of the virus to be condensed. The method is now part of the routine in many Pasteur Institutes. It probably prevents some of the purely toxic effects of the virus in human cases.

Methods.—(1) *Diagnosis.*—The work on the specificity of the Negri bodies for rabies has led to a modification in the procedure to be adopted. Formerly it was advisable, if possible, to keep an animal suspected of rabies alive for the observation of symptoms. While the clinical history of the animal ought to be carefully obtained, greater information will be obtained by examination of its hippocampus. The animal should therefore be killed and the brain removed after reflecting the scalp and cutting through the calvarium with a sharp chisel. The brain is laid down, vertex uppermost, and the upper parts of one hemisphere are removed in thin horizontal slices till the anterior part of the lateral ventricle is reached. The roof of the ventricle is then cut away with a probe-pointed bistoury, and the hippocampus will be recognised as the laterally arched ridge which forms the floor of the ventricle This may be transversely incised and thin slices removed for the making of smears and sections. A portion should also be taken from the cortical grey matter of the cerebrum and from the cerebellum. Smear preparations may be made as follows. A thin portion of nervous tissue is placed on a glass slide towards one end, on this a cover-glass is placed, and then, gentle pressure being exerted, a smear is made by sliding the cover-glass towards the other end of the slide. Smears are allowed to dry in the air and are then fixed in methyl alcohol; they may be then stained by Giemsa's stain or by any eosin-methylene-blue combination. Frothingham recommends the method of making "impression preparations." A slice of tissue, *e.g.* of hippocampus, is placed on a piece of wood, the porosity of which causes it to adhere. A clean slide is then lowered upon the tissue and slight pressure is applied. On raising the slide a thin film of cells preserving their original arrangement is lifted off, and this can be fixed and stained like a smear preparation.

For sections, Zenker's fluid is to be recommended as a fixative; the tissue should be in thin slices and should not be allowed to remain in the fixative for more than four to five hours The tissue is then treated in the usual way (p. 119), and paraffin sections are stained by any of the above-mentioned methods. We have found that the Negri bodies can be quite satisfactorily demonstrated by fixing the tissue in formalin and staining paraffin sections by Leishman's methods.

In addition to microscopic examination, a small piece of the medulla or cord of the suspected animal should be taken, with aseptic precautions, rubbed up in a little sterile 0·75 per cent. sodium chloride solution, and injected by means of a syringe beneath the dura mater of a rabbit, the latter having been trephined over the

cerebrum by means of a small trephine. In rabies in the rabbit, symptoms of paresis usually occur in from six to twenty-three days, and death in fifteen to twenty-five days. When the material for inoculation has to be sent any distance, it is best to pack the head of the animal in ice. The virulence of organs is not lost, however, if they are simply placed in glycerin.

(2) *Treatment.*—Every wound inflicted by a rabid animal ought to be cauterised with the actual cautery or with fuming nitric acid as soon as possible. By such treatment the incubation period will at any rate be lengthened, and therefore there will be better opportunity for the Pasteur inoculation method being efficacious. The person ought then to be sent to the nearest Pasteur Institute for treatment. It is of great importance that in such a case the nervous system of the animal should also be sent, in order that the diagnosis may be certainly verified.

MEASLES

The earliest reliable experimental observations on the etiology of this disease were made by Hektoen in 1905, who reproduced measles in volunteers by the subcutaneous injection of blood from cases at an early stage of the illness. No bacteria could be demonstrated in the blood by culture. Anderson and Goldberger, a few years later, were apparently successful in transmitting the disease to monkeys by inoculation with blood and with nasal and pharyngeal secretions. They found that the causal agent was filterable through Berkefeld filters, and they demonstrated its presence in the blood and throat secretions of inoculated animals. Though some workers have been unable to confirm these results, it seems now well established that a virus is present in the blood at an early stage, which is experimentally transmissible to monkeys, reproducing a condition similar to human measles. Blake and Trask inoculated monkeys (Macacus rhesus) intratracheally with filtered naso-pharyngeal washings from cases at the commencement of the eruptive stage, and produced a condition presenting many similarities to human measles, with lesions of the skin and the mucous membrane of the mouth corresponding histologically to the measles eruption. They were able to pass the infection through a series of animals, and they demonstrated a specific immunity following an attack. According to Duval and D'Aunoy, rabbits can also be infected by the intravenous injection of blood from cases in the early eruptive stage, and they claim that a reaction corresponding to the human disease results, with the development of Koplik spots on the buccal mucosa. They have also produced the same effects with filtered naso-pharyngeal washings. A considerable

amount of evidence is therefore available in favour of the virus of this disease being filterable.

Quite recently, Tunnicliff has described a very minute Gram-positive diplococcus which has been cultivated from the blood and from the nasal, pharyngeal, and conjunctival secretions in the early stage of the disease, including the pre-eruptive stage. This organism has been grown primarily under anaerobic conditions, but can be maintained in aerobic culture. On blood agar the colonies are coloured green, like the " viridans " types of streptococci. According to Tunnicliff, dead cultures yield a skin reaction in susceptible persons analogous to the Dick reaction (*vide* p. 263) ; this is absent, however, in most persons who are immune as a result of an attack of the disease. These observations await confirmation by other workers.

The immunity following an attack of measles has long been recognised, and experimental work has shown that it depends mainly on specific properties resident in the blood serum. The serum of recent convalescents has been used both in the prophylaxis and treatment of measles, with promising results. It has been found that if the immune serum is injected during the first five days after infection, an effective passive immunity is produced which may persist for a month ; according to Degkwitz, 2·5 c.c. serum is the dose for a child under four years of age. Further, an injection even after the fifth day tends to modify the subsequent attack. With a view to providing facilities for the supply of convalescents' serum, centres have been established in certain continental countries and in America for collecting and distributing the serum, which is obtained from convalescent donors over ten years of age (it is necessary, of course, to ensure that they are free from any other systemic infection, *e.g.* syphilis, tuberculosis). The pooled serum of usually three persons is used, but at present there is no means of standardising the serum.

CHAPTER XXVI

FILTER-PASSING VIRUSES (*continued*): EPIDEMIC POLIOMYELITIS, EPIDEMIC ENCEPHALITIS, HERPES

Epidemic Poliomyelitis

While the occurrence of "infantile paralysis" (Heine-Medin disease) of sudden onset, and affecting especially one or more limbs, has been known since the earliest times, it is only coincident with the modern developments of neurology that the most prevalent type has been recognised to be associated with inflammatory changes which are specially concentrated in the anterior cornua of the spinal cord. Though the disease chiefly attacks children, older subjects are also affected, and in some epidemics the infection of adults is a prominent feature. The disease is usually sporadic in its incidence, and, as has long been known, in temperate climates it is of most frequent occurrence during the warmer months of the year. It also occurs in an epidemic form. Such outbreaks have been familiar in Norway and Sweden during the last century, but in other countries similar epidemics, limited or extensive, have come under notice. Thus in New York in the summer of 1907 an outbreak of probably over 2000 cases occurred, 762 of which were carefully investigated by a special Commission, and it is from their work that much of our present knowledge of the disease has been derived, and many facts regarding its infective nature have been definitely established. An even more serious epidemic took place in New York in 1916. Clinically, the onset of the condition is marked by more or less pronounced fever, often accompanied by sore throat and followed after a few days by signs of paresis and paralysis; as a rule, in only a relatively small proportion of cases does death result, though there is a great variation in the mortality in different outbreaks. When recovery occurs, many of the paralytic symptoms may pass off, but generally there remains evidence of definite permanent injury to the motor functions of the nervous system. Pathologically, the initial lesions consist in a local or general leptomeningitis with pro-

nounced leucocytic exudation of a polymorpho-nuclear type into the perivascular lymphatics, the existence of which is reflected in the appearance of such cells in moderate numbers in the cerebro-spinal fluid. In the cord the inflammatory condition is usually marked in the arterioles of the anterior commissure, especially in the cervical and lumbar regions, and thence passes into the anterior cornua along the vessels, which show intense hyperæmia with perivascular cell proliferation, and which may become thrombosed or rupture. The nutrition of the grey matter is thus interfered with, the nerve cells may die and become the prey of neuronophages, and secondary local and systemic degenerations may follow. Such a pathological picture, however, is not confined to the grey matter nor indeed to the cord, as similar changes have been observed in the brain. The recognition of this has widened the whole conception of the disease, and various clinical types besides the classic anterior poliomyelitis are now recognised to exist. These depend partly on variations in the severity of the condition, partly on the disease being concentrated in a particular part of the nervous system. These less common types probably include many cases described as the acute ascending paralysis of Landry, acute bulbar paralysis, cases characterised by acute meningitis or encephalitis, cases of rapidly developing ataxia, and even cases simulating neuritis.

The infectivity of the disease was established by the work of Landsteiner and Popper, who in 1909 in Vienna succeeded in producing the disease in a monkey by the intraperitoneal injection of an emulsion of the spinal cord of a child who had succumbed on the fourth day of illness. Similar observations were made in the same year by Flexner in New York, who found that if for intraperitoneal injection intracerebral inoculation was substituted, positive results were more uniformly produced, and the brain and cord of the infected animals were infective for other monkeys, the incubation period being from four to thirty-three days. It is on the work of Landsteiner, Levaditi, and especially of Flexner, that our present knowledge is chiefly based. Hitherto the monkey is the only animal to which the disease has certainly been communicated—both the anthropoid apes and the lower monkeys are susceptible, and the conditions resulting from inoculation are clinically and pathologically identical with those observed in man. Although rabbits may occasionally develop the disease after experimental inoculation, this is exceptional.

With regard to the nature of the virus, the discovery was made

independently by Flexner and Lewis, and by Landsteiner and Levaditi, that it could pass through an earthenware filter (*e.g.* Berkefeld N or V). The deduction from this observation was that the causal organism must be very small, and Flexner and Noguchi succeeded in cultivating minute bodies which there is reason to suppose are the infective agent. In their experiments, small portions of the central nervous system—preferably the brain—removed *post mortem*, were inserted in a medium composed of naturally sterile ascitic fluid containing a fragment of sterile fresh rabbit kidney, and the cultures incubated at 37° C. under anaerobic conditions. About the fifth day faint opalescence appeared, and the fluid was found, when treated with the Giemsa stain, to contain minute bluish or violet globoid bodies, about 0·2 μ in diameter, in pairs, chains, or, less commonly, in groups. Towards Gram's stain their behaviour was variable. It was found that similar cultures could be raised from the infective filtrates. Further, the organism could adapt itself to other media and could be maintained in subculture. By inoculating monkeys with these cultures, under precautions which excluded the possibility of infection being derived from the brain matter originally used, poliomyelitis was set up in the animals, and the organism was recovered from their brains. The "globoid bodies" (whose nature is unknown) were also microscopically demonstrated, by means of the Giemsa stain, in the brain in both the natural and experimental disease.

In infecting monkeys from a human case it is advisable to commence with the use of an emulsion of the central nervous system, for filtered emulsions possess much less virulence; but after a few *passages* through monkeys it is found that filtration has little effect in diminishing the number of successful inoculations, the virus being now so potent that 0·001 to 0·01 c.c. of an emulsion of material from the central nervous system (p. 640) in distilled water will originate the disease when injected into the brain. Such a virus withstands glycerination for years, and can be kept frozen at −2° to −4° C. without being affected. It also withstands from 1 to 1½ per cent. phenol for at least five days; it is, however, killed by an exposure at 45° to 50° C. for half an hour. The disease can be originated by subdural and intracerebral injection, and also by introduction into the sheath of such a nerve as the sciatic. When the sheath of a nerve is infected, the paralytic symptoms usually first appear in relation to that part of the cord from which the nerve emerges. Infection can also readily be produced by scarifying the mucous membrane of the nose and rubbing the virus into it,

or even by simply injecting it into the nasal cavities. The intraperitoneal, intrathecal, and subcutaneous routes can also be employed, but to cause the disease by intravenous injection enormous doses must be administered. By means of the inoculation method the distribution of the virus in the natural and experimental disease has been determined and has been found to be similar in both cases. The virus is markedly neurotropic, and is highly concentrated in the brain and spinal cord. It also occurs in the intervertebral ganglia, the Gasserian ganglion, and in the abdominal sympathetic ganglia. It may be found in the lymphatic glands, especially the tonsil and those of the mesentery, and it has been demonstrated in the nasal mucous membrane. It is absent from the other solid organs and the blood.

Flexner's view of the pathology of the disease is that infection takes place through the nasal mucous membrane, a catarrh of the buccal and nasal cavities being often the first sign of the disease. In monkeys in which intracerebral inoculation has been practised the virus is eliminated into the nose, and the nasal mucus has been found to be infective in human cases. When an individual is infected by the inhalation of such mucus, it is probable that the virus gains access to the brain by the lymphatics of the olfactory nerves; this view rests on the observation that when monkeys are inoculated by painting the infective material on the nasal mucosa, the olfactory lobe becomes infected before other parts of the brain. This fact, as well as the size of the dose required to produce infection by intravenous injection, militates against the possibility of the virus being carried to the central nervous system by means of the blood under natural conditions. There is evidence from experimental intravenous injections that the choroid plexus, which is the source of the cerebro-spinal fluid, prevents (so long as it is uninjured, *e.g.* by inflammation) the passage of virus into the subarachnoid space. It is likewise possible that in natural infection the virus may pass into the mesenteric nodes and thence be absorbed by the lymphatics of the spinal nerves. All the facts point to the importance of the part played by the peri- and intra-neural lymphatics in the passage of the causal agent to the central nervous system. While the virus may be said to be neurotropic, the term must be used in the sense that all the elements of the nervous system—pia-arachnoid, glia, interstitial blood vessels as well as parenchymatous cells—show a special susceptibility. The pathological anatomy in these structures has been described above.

These observations have furnished important indications of

the method by which infection takes place, and by which both the sporadic cases and the epidemic outbreaks occur. It has been found that in monkeys recovered from the disease, the nasal mucosa remains infective for many months after the virus has disappeared from the central nervous system, and it has been established that in man there are chronic carriers such as exist in other diseases. As in other conditions, the carrier may not himself suffer from the effects of the infective agent which he carries. Further, the occurrence of abortive cases may constitute a means by which infection is maintained in a community. Such abortive cases are probably fairly common during epidemics. In connection with this aspect of the subject, Amoss and Taylor have made the interesting observation that in some individuals the normal nasal secretion possesses a certain power of neutralising the poliomyelitic virus. Finally, it is to be noted that there is a periodicity in the incidence of poliomyelitis in an epidemic form. As bearing on the explanation of this, Flexner, Clark, and Amoss record the case of one strain of the virus the virulence of which in monkeys was at first low, and then rose to a maximum which was maintained for three years; this phase was succeeded by a decrease in infectivity in a few months, without apparent cause. It is obvious that this fact is not only of importance in relation to poliomyelitis, but is suggestive as bearing on the periodicity of other epidemic diseases.

Some workers differ from Flexner on certain points regarding the pathology of poliomyelitis. Thus Rosenow and Towne look on the "globoid bodies" as specially small forms of a rather large streptococcus which they have isolated from the brain in both the natural and experimental disease—which can grow under aerobic conditions and can produce poliomyelitis, not only in monkeys but also in rabbits. These organisms have been found in certain cases by other observers, who, however, deny that the disease conditions they produce are to be classified with poliomyelitis. Another point on which difference of opinion has arisen is regarding the mode of spread of the disease. Rosenau has put forward observations pointing to a blood-sucking fly, *Stomoxys calcitrans*, being capable of transferring the disease in monkeys. As the worst epidemics of poliomyelitis occur in summer, the possibility of an insect carrier has thus been entertained. The absence of the virus from the blood in man, and the difficulty of originating the disease by intravenous injection, are facts militating against such a theory, which, it must be held, has not yet been established.

Though no cases are recorded of a second attack of poliomyelitis in man, our knowledge regarding immunity is mainly derived from animal experimentation. Monkeys which have passed through an attack of the disease are insusceptible to fresh inoculation, but definite disease manifestations are apparently essential to the establishment of immunity, as animals which have at first yielded negative results are usually susceptible to a second inoculation. Both in man and in the monkey the serum of a recovered case contains substances capable of neutralising the virus, for if such serum be mixed with virus and incubated for a time at 37° C. the mixture becomes inoperative on intracerebral injection into monkeys. The antibodies persist in the serum in man for many years after an acute attack, and they possess this further significance, that they may be found in the so-called abortive cases where a transient illness with little or no involvement of the nervous system occurs. The only evidence, in fact, that such a condition is due to the virus of poliomyelitis lies in the fact that subsequently the serum has the capacity of neutralising the virus. Not only has the immune serum neutralising properties *in vitro,* but it has been shown experimentally to have a certain effect *in vivo* when introduced intrathecally into monkeys previous to intravenous inoculation. Further, the serum of recently recovered human cases when injected into patients suffering from poliomyelitis (especially during the first forty-eight hours) has a capacity of arresting paralysis. The amount of serum given has been from 35 to 120 c.c., administered both intrathecally and intravenously. It is stated by Kraus that if the virus which has been killed by exposure to phenol is injected into monkeys they develop resistance, but such a prophylactic vaccine treatment requires further investigation.

The fact that poliomyelitis appears under a variety of clinical types makes the diagnosis difficult in many cases, especially of mild illness. This is specially true of the meningitic type, which may be difficult to distinguish from epidemic cerebro-spinal meningitis, especially as the characters of lumbar puncture fluid in the two diseases are very similar, and, as is known, it may often be difficult to isolate the meningococcus where it is actually the causal organism. It may be stated that cases have occurred where the diagnosis lay between poliomyelitis and the paralytic type of rabies, and in the present stage of knowledge the susceptibility of the rabbit to the latter disease would constitute the only means by which the diagnosis could be arrived at.

Römer has described a paralytic disease in guinea-pigs closely resembling human poliomyelitis.

Methods.—The inoculation of a monkey constitutes the only certain means of diagnosis in a doubtful case of poliomyelitis. A piece of brain removed with all aseptic precaution from a fatal case is ground up with sand and saline (the brain tissue forming 5 per cent. of the whole). The product is centrifuged, and the supernatant fluid, filtered through paper, is used for the inoculation. Portions of the central nervous system may be placed in glycerin when transmission to a laboratory is necessary. In certain cases information might be obtained by inoculating material from swabs of sterile wool allowed to remain in the nasal passages, in order that the mucus may be absorbed. Portions of tissue removed from the tonsils might also be useful; in each case the material may again be immersed in small quantities of glycerin, or advantage may be taken of the fact that the virus can survive exposure to 1 per cent. phenol for several days.

Epidemic Encephalitis (*Encephalitis lethargica*)

During the spring and summer of 1918 a number of cases of encephalitis occurred in Britain which were characterised clinically by lethargy and drowsiness, often passing into coma, with moderate or no rise of temperature. A great variety of nervous symptoms were recorded—headache, epileptic fits, spastic phenomena, ascending paralysis, etc.—but the most common and striking feature was the existence of irritative and paralytic affections of the muscles of the eyelids and eyeballs. The mortality was high. In fatal cases the chief post-mortem changes were small sub-pial hæmorrhages and hæmorrhages into the grey and white matter of the brain. There was sometimes marked subdural œdema. Meningitis was not a marked feature, and when it occurred, was patchy; the cerebro-spinal fluid was usually clear, there being at first a slight lymphocytosis which disappeared later. Microscopically, the hæmorrhages appeared to be of venous origin; there was intense capillary congestion and patchy perivascular round-cell infiltration, as well as focal collections of these cells in the substance of the brain. The lesions affected especially the pons, medulla, and mid-brain; degenerative changes in the oculo-motor centres were recorded. The occurrence of ophthalmoplegia suggested that the condition was botulism, but the symptoms of the two diseases did not otherwise correspond. No association with the taking of particular articles of food was traceable, and no evidence was obtained that B. botulinus was responsible for the condition. It was also suggested that the condition was

poliomyelitis of an aberrant type, but the findings differed in certain respects from those of the cerebral cases which have been observed during epidemics of poliomyelitis, and, further, there was no evidence of a concurrent prevalence in Britain of ordinary poliomyelitis. It is noteworthy that in encephalitis the cellular infiltrations in the brain have been found to consist mainly of lymphocytes, while neutrophile polymorphs are rare, whereas the latter cells are numerous in the corresponding lesions of poliomyelitis. The condition was not confined to Britain, an outbreak having been recorded in Austria during 1917, and in France in 1918; and a disease of a similar nature occurred in 1917 in Australia. In the British cases the bacteriological findings were as a rule negative.

Since encephalitis lethargica was first recognised as an epidemic disease in this country its incidence, as judged by notification tables, has tended to increase, and the condition has been specially prevalent in urban districts. The disease has shown an increased incidence during the first quarter of the year, which contrasts with the seasonal prevalence of epidemic poliomyelitis. The infective nature of the condition has been generally accepted, and attention has been called to the occurrence of mild or ambulatory cases. This has raised the question of infection being spread by "carriers," as in the case of epidemic poliomyelitis. It has been suggested that the infection atrium is the upper respiratory tract. A condition of so-called "epidemic hiccup" which has been reported in recent years has also been regarded as a manifestation of epidemic encephalitis.

Extensive investigations have been made into the nature of the infection, but so far without conclusive results. As a result of experiments in which encephalitis has been reproduced in animals by intracerebral inoculation with material from the human disease (*vide infra*), the virus has been regarded as one of the filter-passers. Loewe and Strauss have reported the cultivation from the disease of minute globoid bodies similar to those described in poliomyelitis (*vide* p. 636), and Rosenow has claimed that the condition is due to a type of streptococcus. These observations have not been confirmed. Various workers have observed minute granular structures (Da Fano's "minute bodies") situated intracellularly in the lesions, similar to those described in experimental herpetic encephalitis (*vide* p. 645). Though it has been suggested by some that these cell inclusions represent the virus, others, *e.g.* Cowdry and Nicholson, hold that the structures in question are merely cell-derivatives. In the interpretation of the results of experimental transmission of the

disease to laboratory animals, a serious fallacy has been brought to light, namely, the occurrence of spontaneous encephalitis in certain of these animals, *e.g.* rabbits and monkeys, from which they may recover, though the histological lesions, in the form of round-cell infiltrations, may persist for some time.

In 1920 Levaditi and Harvier reported that they had transmitted experimentally the virus of encephalitis lethargica and demonstrated its pathogenicity for certain laboratory animals, *e.g.* rabbits, the lesions being the same as in the human subject, and similar results have been reported by McIntosh and others. They claimed that it was filterable, and, like certain other viruses of this nature, could be preserved in glycerin, and concluded that it was conveyed to the central nervous system by cranial, ocular, and peripheral nerve routes, but was not pathogenic if injected subcutaneously, intravenously, or intraperitoneally, or if introduced into the trachea or stomach. McIntosh and Turnbull in the same year claimed to have reproduced the disease in a *Patas* monkey by combined subdural and intraperitoneal inoculation of filtered emulsion of the cervical cord, pons, and basal nuclei from a fatal human case. The animal developed convulsive attacks and ultimately became lethargic and died on the fifty-sixth day. The microscopic lesions in the brain resembled those in the human disease. Lower monkeys, however, are in general insusceptible to experimental infection. It should be noted that certain of the claims made regarding the experimental transmission of the disease have rested only on the finding of microscopic lesions—a fallacious criterion in view of the occurrence of spontaneous encephalitis in experimental animals. Levaditi and his co-workers, and Doerr and Schnabel have shown that the viruses isolated by them from cases of encephalitis produce a characteristic train of symptoms, and the same holds good for the encephalitis-producing virus isolated by Flexner (*vide infra*). These symptoms consist of pyrexia, uncontrolled muscular movements, gnashing of the teeth, retention of urine, convulsions and paralysis, and death within seven days, but such effects are identical with those produced by a neurotropic strain of the herpes virus (*vide* p. 644), and on this basis it has been suggested that the viruses of the two diseases are closely related. Attempts to cultivate these viruses by various methods have proved unsuccessful (McCartney). If we exclude as inconclusive the experiments in which the sole evidence of disease in the inoculated animals has been the finding of microscopic lesions in the brain, and accept as the only reliable criterion the symptom-complex

referred to above, then of the many attempts to transmit encephalitis with material from the human subject very few have been successful. It is worthy of note that Flexner and Amoss produced encephalitis in rabbits by intracerebral injection of the cerebro-spinal fluid from a case of neuro-syphilis which showed no evidence of encephalitis. Again, the existence of a herpes virus known to produce the same effects as the supposed encephalitis lethargica viruses adds a further difficulty in the interpretation of experimental results. Further facts of importance are that the viruses derived from cases of encephalitis also produce herpetic keratitis in rabbits, and that, as shown by Levaditi in cross immunity experiments, the virus of herpes febrilis and his supposed encephalitis virus are identical. It seems likely that the herpes virus, undoubtedly widely prevalent, may become generally distributed in the tissues and fluids of the body. Thus, if all the facts are taken into consideration, the possibility must be considered that the virus recovered from cases of encephalitis lethargica is merely the herpes virus. On the other hand, the view that encephalitis and herpes in the human subject are merely manifestations of the action of the same virus can hardly be accepted on the evidence at present available.

Spontaneous Encephalitis of Rabbits.—This condition, first noted by Bull and subsequently studied by Oliver and by Twort and Archer, has been referred to above, and requires consideration in relation to the problem of epidemic encephalitis. It may be noted that the condition is usually associated with nephritis. It is readily transmitted both by cage infection and by experimental inoculation. The disease may be widespread. McCartney, working in America, found that about one-half of the stock rabbits examined by him showed encephalitis lesions, *e.g.* perivascular, meningeal, and sub-ependymal areas of round-cell infiltration and necrotic foci. It is of particular interest that Levaditi, Nicolau, and Schoen have described in this condition a micro-organism classified among the microsporidia and designated *Encephalitozoön cuniculi*. This organism is found next to the subcortical areas of infiltration and occurs in groups of twenty to forty exceedingly small elongated or pear-shaped structures apparently enclosed in a cyst.

Herpes

Within recent years the etiology of herpes has been extensively studied, and it has been proved that the vesicular fluid of certain types of herpes (*e.g.* herpes febrilis, cornealis, genitalis) contains an infective agent or virus which some observers have claimed to be filterable. The evidence in regard to the infective nature of herpes zoster has been less conclusive than in the case of the other types. Grüter and Löwenstein first showed that the

vesicular fluid of herpes febrilis was infective for rabbits when inoculated on the cornea, producing a herpetic keratitis similar to herpes cornealis in the human subject, the infection being transmissible in a series of animals. The virus was rendered inactive by exposure to a temperature of 56° C. for half an hour or by being kept outside the body at 37° C. for twenty-four hours. The experimental production of herpetic keratitis in rabbits by local inoculation with the vesicular fluid of various forms of herpes has been confirmed by subsequent workers. Also, it has been shown that material from the lesions in the rabbit, when inoculated into the skin or cornea in man, reproduces the typical herpetic vesicles. The herpes virus apparently possesses little capacity to attack the nervous system in the human subject, but its virulence for susceptible animals may be considerable. Thus, while the experimental keratitis in rabbits may ultimately clear up without any further effects, in a certain proportion of experiments, as first shown by Doerr and his co-workers, the virus attacks the central nervous system and produces meningo-encephalitis with death of the animal. The symptoms comprise pyrexia, muscular incoordination, salivation, retention of urine, teeth-gnashing, dashing movements, loss of equilibrium, and finally convulsions and paralysis. The changes in the central nervous system are characterised by lymphocytic and leucocytic infiltrations, marked nerve-cell degeneration and proliferations of fixed tissue cells. This experimental condition has proved of great interest in view of its similarity to the changes in the brain in epidemic encephalitis and in animals inoculated with material from cases of this disease. A general herpetic infection in the rabbit has also been produced by intravenous injection of the conjunctival secretion of an animal with experimental keratitis (Doerr and Vöchting). It has been shown that the subdural inoculation in rabbits of material containing the virus either from the human subject or experimental keratitis produces a rapidly fatal encephalitis, and this condition is then transmissible in a series of animals by subdural inoculation with brain emulsion; further, corneal inoculation with brain emulsion produces a herpetic keratitis and in some cases encephalitis (Blanc and others). The herpetic keratitis of rabbits is also transmissible to guinea-pigs (Doerr and Vöchting and others). The vesicle fluid inoculated by scarification on other sites in the same individual may reproduce a herpetic condition. The virus has also been found in the blood and the spleen of experimentally infected animals; and in a human case the cerebro-spinal fluid has been shown to be infective for rabbits (Ravaut and Rabeau).

Using as the inoculum the vesicle fluid of herpes zoster, Lipschütz succeeded in producing a transmissible keratitis in rabbits, and Goodpasture and Teague have claimed to have reproduced herpes zoster by inoculating a herpes simplex virus into the skin of rabbits and guinea-pigs after painting with tar.

The relationship of the herpes and epidemic encephalitis viruses has been suggested in view both of the experimental production of encephalitis by known herpes viruses and also of Levaditi and Harvier's observation that, by means of brain emulsion from rabbits infected with a supposed epidemic encephalitis virus, experimental keratitis could be produced followed by typical encephalitis. In fact, Levaditi, Harvier and Nicolau concluded that the herpes and encephalitis viruses were identical, the former being only a less virulent type of the latter. This has been considered above in regard to the etiology of epidemic encephalitis. Apparently the herpes virus may spread along the axis cylinders of motor and sensory nerves from the periphery to the central nervous system, and in the brain and cord the resulting lesions may be anatomically related to the nerves by which the virus has entered (Goodpasture). A rabbit which has recovered from experimental herpetic keratitis is immune to further inoculation. At first the immunity is most marked in the cornea of the same eye, but later the other cornea becomes insusceptible to inoculation, and this refractory state becomes intensified over a period of several months. After recovery from corneal infection the animal is insusceptible also to intracerebral inoculation with herpes virus. According to Blanc, Tsiminakis and Caminopetros, the serum of recovered animals (or of human cases of encephalitis) does not neutralise the virus when added *in vitro*. Perdrau has found that cerebral immunity to the herpes virus may follow the intradermal inoculation of a neurotropic virus, though if the inoculation is deep (*e.g.* by deep scarification), encephalitis may result. The immunity persists for three months. According to Flexner and Amoss, strains of the virus vary considerably in virulence; those of low virulence only produce encephalitis when inoculated intracerebrally. Further, by inoculating with a mild virus, immunity to the more virulent strains may be produced. There is also evidence that viruses of this type may be present in the saliva of healthy persons (Levaditi, Harvier and Nicolau; Doerr and Schnabel).

With regard to the biological nature of the herpes virus little is known and its supposed filterability has been questioned. As in epidemic encephalitis, minute granular structures (intra-

cellular and extracellular) have been described in the lesions of the central nervous system which some observers have regarded as specific and have identified with the virus. These bodies can be demonstrated by Giemsa's stain. Peculiar eosinophile cell inclusions in the corneal lesions have been observed by Luger, Lipschütz, Levaditi, and others. At the present time it is impossible to express a definite opinion as to the nature of these bodies, but they are probably to be regarded as analogous to the vaccinia bodies (p. 620). Attempts to cultivate the virus have proved practically unsuccessful, though McCartney has been able to maintain the herpes virus in a culture medium consisting of unheated rabbit brain extract, through three subcultures.

CHAPTER XXVII

PROTOZOAL DISEASES: MALARIA, AMŒBIC DYSENTERY

Malaria

It has long been established that the malarial fevers are protozoal infections, there being different species of the parasite. These belong to the Hæmosporidia (a sub-class of the Sporozoa), which are blood parasites, infecting the red corpuscles of mammals, reptiles, and birds. The parasite is known by the generic name, *Plasmodium*; the term *Hæmamœba* has also been employed. It was first observed by Laveran in 1880, and his discovery received confirmation from the independent researches of Marchiafava and Celli, and later from the researches of many others in various parts of the world. Golgi supplied important additional information, especially in relation to the sporulation of the organism and the varieties in different types of malarial fever. In this country valuable work on the subject was done by Manson, and to him specially belongs the credit of regarding the exflagellation of the organism as a preparation for an extra-corporeal phase of existence. By induction he arrived at the belief that the cycle of existence outside the human body probably took place in the mosquito. It was specially in order to discover, if possible, the parasite in this insect, that Ross commenced his long series of observations, which were ultimately crowned with success. After patient and persistent search, he found rounded pigmented bodies in the wall of the stomach of a dapple-winged mosquito (a species of Anopheles) which had been fed on the blood of a malarial patient. The pigment in these bodies was exactly similar to that in the malarial parasite, and he excluded the possibility of their representing anything else than a stage in the life-cycle of the organism. He confirmed this discovery and obtained corresponding results in the case of the Proteosoma infection of birds, where the parasite is closely related to that of malaria. In birds affected with this organism, he was able to trace all stages of its development, from the time it entered

the stomach along with the blood, till the time when it settled in a special form in the salivary glands of the insect. Ross's results were published in 1898. Exactly corresponding stages were afterwards found in the case of the different species of the human parasite, by Grassi, Bignami, and Bastianelli; and these with other Italian observers also supplied important information regarding the transmission of the disease by infected mosquitoes. Abundant additional observations, with confirmatory results, were supplied by Koch, Daniels, Christophers, Stephens, and others. Wherever malaria has been studied the result has been the same. Lastly, we may mention the striking experiment carried out by Manson by means of mosquitoes fed on the blood of patients in Italy suffering from mild tertian fever. The insects, after being thus fed, were taken to London and allowed to bite the human subject, Manson's son, Dr. P. Thurburn Manson, offering himself for the purpose. The result was that infection occurred; the parasites appeared in the blood, and were associated with an attack of tertian fever. Ross's discovery has not only been a means of elucidating the mode of infection, but, as will be shown below, has also supplied the means of successfully combating the disease.

From the zoological point of view the mosquito is regarded as the definitive host of the parasite, the human subject as the intermediate host. But in describing the life-history, it will be convenient to consider, first, the cycle in the human body, and, secondly, that in the mosquito. We shall first give a general account of the life-history and then describe the features of the different species.

The Asexual Cycle in the Human Subject—Schizogony. With regard to this cycle (Plate V., Fig. 21 *a–i*), it may be stated that the parasite is conveyed by the bite of the mosquito in the form of a small filamentous cell—*sporozoite*, which penetrates a red corpuscle and becomes a small amœboid organism or trophozoite. There is then a regularly repeated asexual cycle of the parasite in the blood, the length of which cycle determines the type of the fever. During this cycle there is a growth of the *trophozoites* within the red corpuscles up to their complete development; schizogony then occurs. The onset of the febrile attack corresponds with the stage of schizogony and the setting free of the *merozoites*, *i.e.* with the production of a fresh generation of parasites. These soon become attached to, and penetrate into, the interior of the red corpuscles, becoming intra-corpuscular trophozoites; the cycle is thus completed. The parasites are most numerous in the blood during the

development of the pyrexia, and, further, they are also much more abundant in the internal organs than in the peripheral blood; in the malignant type, for example, the process of schizogony is practically confined to the former.

In addition to these forms which are part of the ordinary asexual cycle, there are derived from the trophozoites other forms, called *gametocytes*, or sexual cells, which tend to be produced especially when the infection has lasted for some time. These remain unaltered during successive attacks of pyrexia, and undergo no further change until the blood is removed from the human body. In the simple tertian and quartan fevers (*vide infra*) the gametocytes are rounded in form, resembling somewhat in appearance the fully developed trophozoites before schizogony, whereas in the malignant type they have a characteristic crescent-like or sausage-shaped form; hence they are often spoken of as "crescents" (Plate V., Fig. 22 *f*, *g*).

The various forms of the parasite seen in the human blood may now be described more in detail.

1. *The Merozoites* are the youngest and smallest forms resulting from the segmentation of the adult amœbula or *schizont*. They are of round or oval shape and of small size, usually not exceeding 2 μ in diameter; the size, however, varies somewhat in the different types of fever. A nucleus and peripheral protoplasm can be distinguished (Fig. 177). The former appears as a small rounded body which usually remains unstained, but contains a minute mass of chromatin which stains a deep red with the Romanowsky method, the peripheral protoplasm being coloured fairly deeply with methylene-blue. The merozoites show little or no amœboid movement; at first free in the plasma, they soon attack the red corpuscles, within which they become the trophozoites. If the blood, say in a mild tertian case, be examined in the early stages of pyrexia, one often finds at the same time schizonts, free merozoites, and the young trophozoites within the red corpuscles.

2. *Intra-corpuscular Trophozoites.*—These include the parasites which have invaded the red corpuscles. They usually occur singly in the red corpuscles, but sometimes two or more may be present together. As seen in fresh blood, the youngest or smallest forms are minute colourless specks, of about the same size as the merozoites; they exhibit more or less active amœboid movement, showing marked variations in shape. The amount and character of the amœboid movement varies somewhat in different types of fever. As they increase in size, pigment appears in their interior as minute dark brown or black specks,

and gradually becomes more abundant (Figs. 172–174 ; Plate V., Fig. 21 *c*, *d*, *e*, Fig. 22 *e*). This pigment is elaborated from the hæmoglobin of the red corpuscles, the parasite growing at the expense of the latter. The red corpuscles thus invaded may remain unaltered in appearance (quartan fever), or may become swollen and pale (tertian fever). In stained specimens a nucleus may be seen in the parasite as a pale spot containing chromatin which may be arranged as a single concentrated mass or as several separated granules, the chromatin being coloured a deep red by the Romanowsky method. The protoplasm of the parasite, which is coloured of varying depth of tint with methylene-blue, shows great variation in configuration (Fig. 174). The young parasites not infrequently present a " ring-form," a portion of the red corpuscle often appearing to be enclosed by the parasite. These ring-forms are met with in all the varieties of the parasite, but they are especially common in the case of the malignant parasite, where they are of smaller size and of more symmetrical form than in the others (Fig. 178).

Within the red corpuscles the parasites gradually increase in size till the mature form (*schizont*) is reached (Fig. 175). In this stage the parasite loses its amœboid movement more or less completely, has a somewhat rounded form, and contains a considerable amount of pigment. In the malignant form it only occupies a fraction of the red corpuscle. The adult parasites may then undergo schizogony, but not all of them do so ; some become degenerated and ultimately break down.

3. *Schizonts.*—In the process of schizogony the nuclear outline becomes lost, and the chromatin becomes divided into a number of small granules which are scattered through the protoplasm ; the latter then undergoes corresponding segmentation and the small merozoites result. The pigment during the process becomes aggregated in the centre and is surrounded by a small quantity of residual protoplasm. (Schaudinn stated in the case of the tertian parasite that schizogony begins by a sort of primitive mitosis, which is then followed by simple multiple fission.) The merozoites are of rounded or oval shape, as above described, and are set free by the rupture of the envelope of the red corpuscles. The pigment also becomes free and may be taken up by leucocytes, the merozoites being free from pigment. The number and arrangement of the merozoites within the schizont vary in the different types. In the quartan there are 6–12, and the segmentation is in a radiate manner, giving rise to the characteristic daisy-head appearance ; in the tertian they number 15–20 or more, and have a somewhat

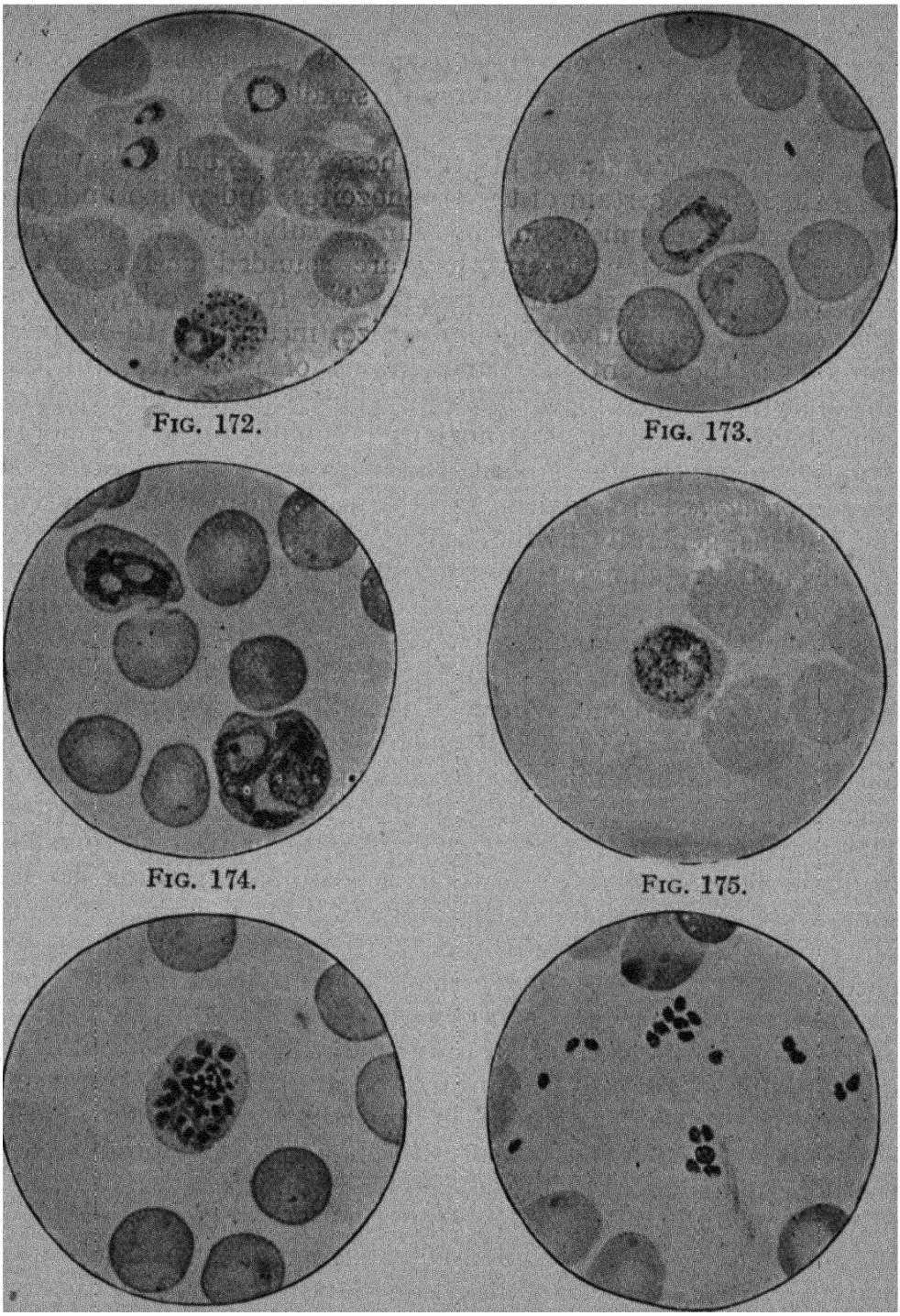


FIG. 172. FIG. 173.

FIG. 174. FIG. 175.

FIG. 176. FIG. 177.

FIGS. 172–177.—Various phases of the benign tertian parasite.

FIG 172. Several young ring-shaped amœbulæ within the red corpuscles, one of the latter enlarged and showing a dotted appearance. Fig. 173. A larger amœbula containing pigment granules. Fig. 174. Two large amœbulæ, exemplifying the great variation in form. Fig. 175. Large amœbula assuming the spherical form and showing isolated fragments of chromatin—preparatory to schizogony. Fig. 176. Schizont, which has produced eighteen merozoites, each of which contains a small collection of chromatin. Fig. 177. A number of merozoites which have just been set free in the plasma. × 1000.

rosette-like arrangement (Fig. 176); in the malignant there are usually 6–25 or more merozoites of small size and somewhat irregularly arranged.

Gametocytes.—As stated above, these are sexual cells which are formed from certain of the trophozoites, and which undergo no further development in the human subject. In the mild tertian and quartan fevers they are rounded and resemble somewhat the largest trophozoites. The female cells, *macrogametocytes*, are relatively of large size, measuring 12–14 μ in diameter; they contain coarse grains of pigment, and the protoplasm stains somewhat deeply blue with the eosin-methylene-blue stains, while the nucleus is small and is commonly situated near the margin of the parasite. The male cells, *microgametocytes*, are smaller, and the protoplasm stains faintly; the nucleus, generally in the centre, is large and often forms a broad band stretching across the cell. In the malignant fevers the gametocytes have the special crescentic or sausage-shaped form mentioned above. They measure 9–14 by 2–3 μ, and occasionally a fine curved line is seen joining the extremities on the concave aspect, which represents the envelope of the red corpuscle (Fig. 179). They are colourless and transparent, and are enclosed by a distinct membrane; in the central part there is a collection of pigment and granules of chromatin. The male crescents (Plate V., Fig. 22 *f*) can be distinguished from the female (*ibid.*, *g*) by their appearance; the former are somewhat sausage-shaped, the pigment is less dark and more scattered through the cell, and there are several granules of chromatin; the latter have more pointed ends and their substance stains more deeply with the blue, the pigment is dark and concentrated, often in a small ring, and there are one or two masses of chromatin in the centre of the crescent. According to the Italian observers, the early forms of the crescents are somewhat fusiform in shape and are produced in the bone-marrow. The fully developed crescents do not appear in the blood till several days after the onset of the fever, and they may be found a considerable time after the disappearance of the pyrexial attacks; they are most numerous in cases in which the infection has lasted for some time. They are also little, if at all, influenced by the administration of quinine. Ross and Thomson have enumerated directly (p. 662) the malarial parasites in the blood at different stages of the disease, and have found that a certain relationship exists between the asexual and the sexual forms, a rise in the number of the former being followed eight to ten days later by a rise in the number of

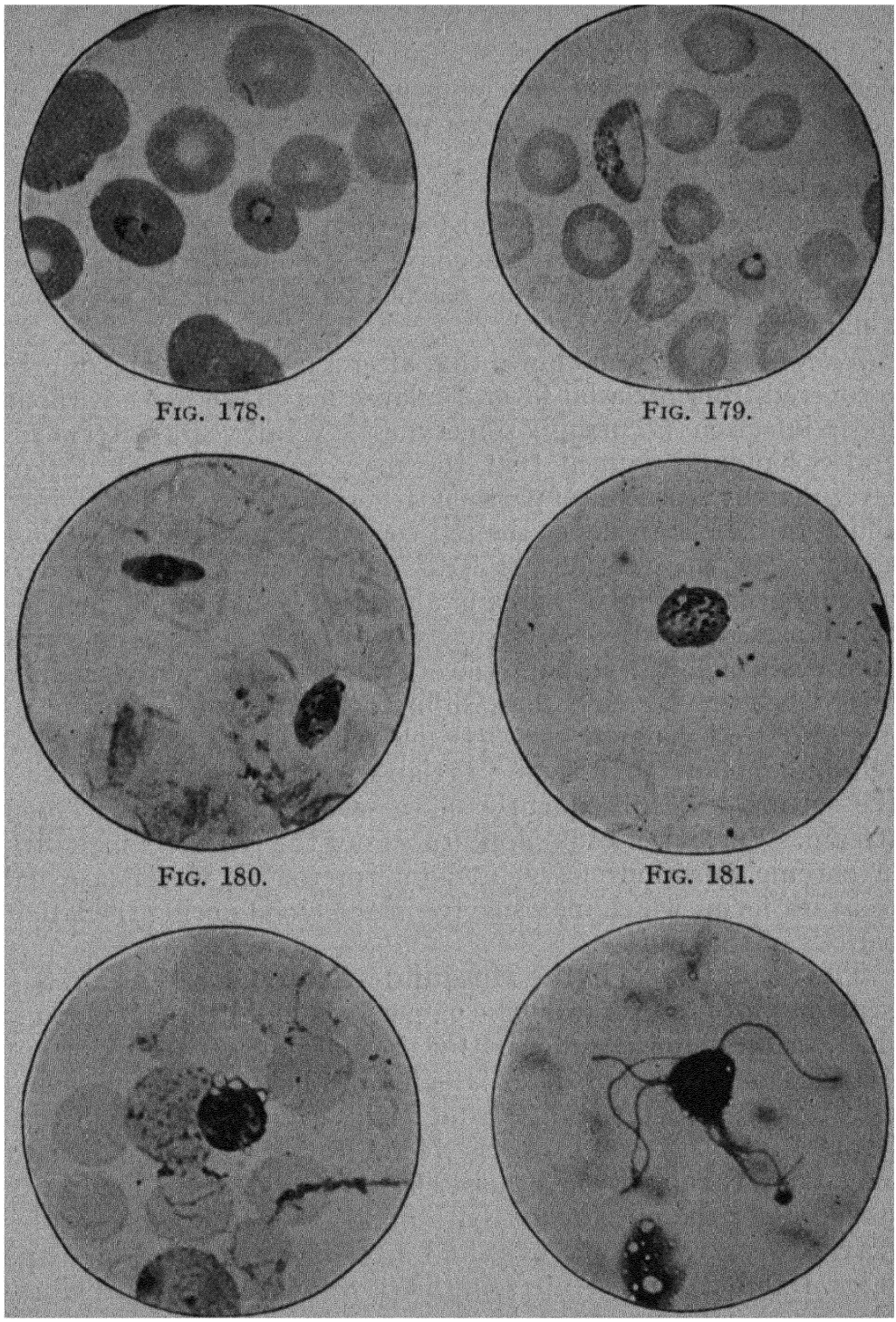


FIG. 178.

FIG. 179.

FIG. 180.

FIG. 181.

FIG. 182.

FIG. 183.

FIGS. 17[illegible]—Exemplifying phases of the malignant parasite.

FIG. 178. Two small ring-shaped amœbulæ within the red corpuscles. Fig. 179. A "crescent" or gamete showing the envelope of the red corpuscles; also an amœbula. Figs. 180–183 illustrate the changes in form undergone by the crescents outside the body. In the interior of the spherical form in Fig. 182 evidence of the flagella can be seen. Fig. 183. A male gametocyte which has undergone exflagellation, showing the thread-like microgametes or spermatozoa attached at the periphery. ×1000. (The figures in this [illegible] are from preparations kindly lent by the late Sir Patrick Manson.)

the latter; they accordingly consider that this is probably the period necessary for the development of the sexual forms. They consider also that the long persistence of crescents in the blood after the fever has ceased, is due not to the long survival of individual crescents, but to their being constantly replenished from asexual forms which persist in the blood and pass through the ordinary process of schizogony, fever only occurring when the number of asexual forms reaches some hundreds per cubic millimetre.

Relapses.—It is well known that after a patient has apparently recovered from malarial fever a relapse may take place without fresh infection occurring, sometimes several years afterward, and Schaudinn claimed that the macrogametocyte of tertian fever may by a process of parthenogenesis give rise to merozoites, which in their turn infect the red corpuscles and start the cycle again. The observations of Ross and Thomson, just referred to, support the view of Bignami and others, that the occurrence of relapses depends on the survival of asexual forms in small numbers, which pass through the ordinary cycle and only produce fever when they again become sufficiently numerous. It is possible also, that malarial parasites may penetrate into endothelial cells and remain latent there; a relapse would then coincide with the setting free subsequently of these organisms in the blood stream. Certain unusual appearances of the parasites met with in chronic infections have been interpreted as special asexual resistant forms which may survive in the blood during the latent periods of the disease.

The Sexual Cycle in the Mosquito—Sporogony.—As already explained, this starts from the gametocytes. After the blood is shed, or after it is swallowed by the mosquito, two important phenomena occur, namely, (*a*) the full development of the sexual cells or gametocytes, and (*b*) the fertilisation of the female cell (Plate V., Fig. 21 *m–q*). If the blood from a case of malignant infection be examined in a moist chamber, preferably on a warm stage, under the microscope, both male and female gametocytes may be seen to become oval and afterwards rounded in shape (Figs. 180–182). Thereafter, in the case of the male cell, a vibratile or dancing movement of the pigment granules can be seen in the interior, and soon several flagellum-like structures shoot out from the periphery (Fig. 183). They are of considerable length but of great fineness, and often show a somewhat bulbous extremity. By the Romanowsky method they have been found to contain a delicate filament of chromatin. They represent the male cells proper, and are known as *microgametes*. They become

detached from the sphere and move away in the surrounding fluid. In the female cell, which has also assumed the rounded form, maturation takes place by the extrusion of part of the nuclear chromatin, this process corresponding to the formation of polar bodies. Fertilisation (syngamy) occurs by the entrance of a microgamete, the chromatin of the two cells afterwards becoming fused. Similar changes occur in the gametocytes of the mild fevers, but, as has been said, the cells are rounded from the first. The fertilised female cell is now generally spoken of as a *zygote* or *oökinete*.

It has been established that the phenomena just described occur within the stomach of the mosquito, and that the fertilised cell or zygote which becomes elongated and motile, penetrates the stomach wall and settles between the muscle fibres ; on the second day after the mosquito has ingested the infected blood, small rounded cells about 6 to 8 μ in diameter, and containing clumps of pigment, may be found in this position. (It was, in fact, the character of the pigment which led Ross to believe that he had before him a stage in the development of the malarial parasite.) A distinct membrane called a *sporocyst* forms around the zygote, and on subsequent days a great increase in size takes place, the cysts coming to project from the surface of the stomach into the body cavity. The zygote divides into a number of cells called *sporoblasts*, and these again divide and form a large number of filiform cells which have a radiate arrangement ; these were called by Ross " germinal rods," but are now usually known as *sporozoites*. The full development (*sporogony*) within the sporocyst occupies over seven days, the length of time depending on the temperature. When fully developed the cyst measures about 60 μ in diameter, and appears packed with sporozoites. It then bursts, and the latter are set free in the body cavity. A number settle within the large veneno-salivary gland of the insect, and are thus in a position to be injected along with its secretion into the human subject. The sporozoites enter red corpuscles and become trophozoites, as above described. Daniels found that, in the case of the malignant parasite, an interval of twelve days at least intervened between the time of feeding the mosquito and the appearance of the sporozoites in the gland.

It will thus be seen that in the human subject the parasite passes through an indefinite number of regularly recurring asexual cycles, with production of collateral sexual cells, and that in the mosquito there is one cycle which may be said to start with the fertilisation of the female gamete.

Varieties of the Malarial Parasites.—The view originally propounded by Laveran was that there is only one species of malarial parasite, which is polymorphous, and presents slight differences in structural character in the different types of fever. It may, however, be accepted that there are at least three distinct species which infect the human subject. Practically all are agreed as to a division into two groups, one of which embraces the parasites of the milder fevers—" winter-spring " fevers of Italian writers—there being in this group two distinct species, for the quartan and tertian types respectively ; whilst the other includes the parasites of the severer forms—" æstivo-autumnal " fevers, malignant or pernicious fevers of the tropics, or irregularly remittent fevers. Formerly Italian writers distinguished several varieties of the latter parasite, though the morphological differences described were slight. Further observations, however, threw doubt on this distinction, and the evidence tended to show that there is a single species. Opinion also varied as to the cycle of this parasite : according to some observers, twenty-four hours, according to others forty-eight hours ; though there is more evidence in support of the latter view, and the term " malignant tertian " or " sub-tertian " is frequently used. The fever is often of an irregular type and multiple infection is probably common. We may therefore speak of three species of human parasites : *Plasmodium vivax* of benign tertian malaria, *Plasmodium malariæ* of quartan malaria, and *Plasmodium falciparum* (*Laverania malariæ*) of malignant or sub-tertian malaria.

We shall now give the chief distinctive characters of the three human parasites :

1. *The Parasite of Benign Tertian Fever* (*P. vivax*).—The cycle of development is completed in forty-eight hours, though a quotidian type of fever may be produced by double infection. The trophozoites have a less refractile margin than in the quartan type, and are thus less easily distinguished in the fresh blood ; the amœboid movements are, however, much more active, while longer and more slender processes are given off. The infected corpuscles become swollen and pale, and may show deeply stained points by the Romanowsky method—" Schüffner's dots." The pigment within the parasite is fine and of yellowish-brown tint. The mature schizont is rather larger than in the quartan, has a rosette appearance, and gives rise to from fifteen to twenty merozoites, though sometimes even more occur ; these have a somewhat oval shape.

2. *Parasite of Quartan Fever* (*P. malariæ*).—The cycle of development in man is seventy-two hours, and produces pyrexia

every third day; double or triple infection may, however, occur. In fresh specimens of blood the outline is more distinct than that of the tertian parasite, and amœboid movement is less marked. Only the smaller forms show movement, and this is not of active character. The infected red corpuscles do not become altered in size or appearance, and the pigment within the parasite is in the form of coarse granules, of dark brown or almost black colour. The fully developed schizont has a "daisy-head" appearance, dividing by regular radial segmentation into from six to twelve merozoites, which, on becoming free, are rounded in form.

In both the quartan and tertian fevers all the stages of development can be readily observed in the peripheral blood. The gametocytes have a rounded form as described above.

3. *The Parasite of Malignant or Sub-tertian Fever* (*P. falciparum*).—The cycle in the human subject probably occupies forty-eight hours, though this cannot be definitely stated to be always the case (*vide supra*). The trophozoites in the red corpuscles are of small size, and their amœboid movements are very active; they often, however, pass into the quiescent ring form (Fig. 178). The pigment granules, even in the larger forms, are few in number and very fine; the infected red corpuscles may be unaltered or tend to shrivel and assume a deeper or coppery tint, sometimes they are swollen and decolorised. The young forms are often found at the edge of the red cells and are elongated ovals or streaks; the rings frequently project from the margin of the corpuscle. The fully developed schizont usually occupies less than half the red corpuscle, and gives rise to from six to twenty or more merozoites, somewhat irregularly arranged and of minute size. Schizogony takes place almost exclusively in the internal organs, spleen, etc. Usually no schizonts can be found in the blood taken in the usual way, but they may be observed in the very severe or pernicious type of the disease. The proportion of infected red corpuscles is also much larger in the internal organs. The gametocytes have the crescentic form, as already described.

Cases of infection with the malignant parasite sometimes assume a pernicious character, and then the number of organisms in the interior of the body may be enormous. In certain fatal cases with coma the cerebral capillaries appear to be almost filled with them, many parasites being in process of schizogony; and in so-called algid cases, characterised by great collapse, a similar condition has been found in the capillaries of the omentum and intestines. The process of blood destruction,

present in all malarial fevers, reaches its maximum in the malignant class, and the brown or black pigment elaborated by the parasites—in part after being taken up by leucocytes, chiefly of the mononuclear class—becomes deposited in various organs, spleen, liver, brain, etc., especially in the endothelium of vessels and the perivascular lymphatics. In the severer forms also brownish-yellow pigment is apparently derived from liberated hæmoglobin, and accumulates in various parts, especially in the liver cells ; most of this latter gives the reaction of hæmosiderin.

Cultivation.—Bass and Johns succeeded in obtaining growths of the parasites of tertian and malignant fevers outside the body. The first cultures were obtained in defibrinated blood from malarial patients, to which was added 1 per cent. of a 50 per cent. solution of dextrose in water. Growth of the parasites took place within the red corpuscles, but only under anaerobic conditions, so that a layer of serum at least half an inch in depth above the sedimented corpuscles was necessary. Under such circumstances, the parasites underwent enlargement and afterwards passed through the stage of schizogony. The merozoites after becoming free are destroyed by leucocytes, but if measures are taken to prevent the presence of these, other generations of growth may be obtained in similarly prepared tubes of blood with sufficient serum. The parasites flourish only in the superficial layers of the sedimented corpuscles, and the most suitable temperature is 37°–41° C. Cultivation of the malaria organisms has since been effected by Thomson and M'Lellan and others. According to J. G. and D. Thomson, removal of the leucocytes is unnecessary. These observers found that in cultures the malignant parasites showed a tendency to clump before and during the stage of sporulation ; on the other hand the benign tertian parasites did not clump.

General Considerations.—The development of the malarial parasites in the mosquito and infection of the human subject through the bites of this insect, have, by the work of Ross and others, as detailed above, become definitely established. These facts, moreover, point to certain definite methods of prevention of infection, which have to a certain extent already been practically tested. The extensive observations carried out go to show that all the mosquitoes which act as hosts of the parasite belong to the genus *anopheles* ; of these there are a large number of species, and in at least eight or nine the parasite has been found. Some of these anopheles occur in England, especially in regions where malaria formerly prevailed. The opportunity for infection from cases of malaria returning from the tropics

to this country thus exists, and such infection has occurred. The breeding-places of the insects are chiefly, though not exclusively, in stagnant pools and other collections of standing water, and accordingly the removal, where practicable, by drainage of such collections in the vicinity of centres of population, the covering in of wells, etc., and the killing of the larvæ by petroleum sprinkled on the water, have constituted the most important measures in localised areas. This procedure has been carried out in various places, for example, in Freetown and Ismailia, with marked success. Also in more open waters fish have been used for the control of mosquitoes. On the other hand, where there are large populous areas, as in India, it has been found almost impracticable to carry out such measures efficiently. Another method is the protection against mosquito bites by netting, it being fortunately the habit of the anopheles rarely to become active before sundown. The experiments of Sambon and Low in the Campagna proved that individuals using these means of protection may live in a highly malarial district without becoming infected. The administration of quinine to persons living in highly malarial regions, in order to *prevent* as well as to treat infection, has also been recommended and carried out, and there appears to be general agreement that in India the properly controlled administration of quinine must, in the meantime at least, be the chief means of combating the disease. In the tropics the natives in large proportion suffer from malarial infection, and one would accordingly expect that infection of the mosquitoes in the neighbourhood of native settlements would be common. This has been found to be actually the case, and it has accordingly been suggested that the dwellings of whites should as far as possible be at some distance from the native centres of population. So far as is known, none of the lower animals have been found to take the place of man as intermediate host to the parasites of malaria.

It may be mentioned, although not bearing on the natural modes of infection, that the disease can also be communicated from one person to another by injecting the blood containing the parasites. Several experiments of this kind have been performed (usually about ½ to 1 c.c. of blood has been used), and the result is more certain in intravenous than in subcutaneous injection. In such cases there is an incubation period, usually of from seven to fourteen days, after which the fever occurs; the same type of fever is reproduced as was present in the patient from whom the blood was taken. This method of inoculation has been applied in recent years for the

treatment of general paralysis, and it has been claimed that malarial attacks artificially produced in this way exert a beneficial effect in this disease.

The Pathology of Malaria.—While much work has been done on the malarial parasite, relatively less attention has been directed to the processes by which it produces its pathogenic effects. It may be said that the organisms are not always equally prevalent in the circulating blood, and at certain stages tend to be confined in the internal organs. Some of the pathogenic effects are associated with particular stages in the life-cycle. Thus the pyrexia occurs when the stage of schizogony is actively in progress, but nothing definite is known as to its real nature and mode of production. We can better explain the anæmia which is so pronounced in cases where the disease is of long standing, and which is due to the actual destruction of red blood corpuscles. The parasite in its sojourn in these cells absorbs their pigment and thus destroys their function; this is further evidenced by the activity displayed by the red marrow in its attempts to make good the loss sustained by the blood. One of the most interesting events in malaria, and one that links it with bacterial infections, is the reaction of the leucocytes. It has been shown that during the apyrexial stages the total number of leucocytes varies greatly, but that there is always an increase of the mononuclear cells, these frequently numbering 20 per cent. or more of the whole, and sometimes even outnumbering the polymorphs. This is such an important feature that in cases where the parasites themselves cannot be demonstrated in the blood, the mononuclear reaction along with the presence of pigment in the mononuclear cells (due to phagocytosis of pigmented parasites and pigment liberated after schizogony) has been taken as evidence that the case is really one of malaria. The mononuclear reaction is specially interesting from the fact that in other protozoal diseases an activity of the same elements has been observed.

The question of immunity to malaria is one of great importance. With regard to Europeans developing immunity, it is difficult to speak. In such a malaria-stricken region as the West Coast of Africa, the death-rate in residents of more than four years' standing is less than in the previous years, but this may be due to the survival of the more resistant immigrants. But there can be little doubt that malaria in the negro is a much less serious condition than in the European. Koch from his observations in New Guinea attributed this to the infection of the native children leading to the development of immunity in the adult community.

He found, what had been independently noted by Stephens and Christophers in West Africa, that the greater number of the children harboured malarial parasites in their blood. The widespread presence of parasites in children might appear to preclude the possibility that the immunity of the adult is due to survival of the most resistant; but the infant mortality in these regions may be very high, and such a survival may be the real explanation. On the other hand, Koch stated that while an immunity appears to exist in native adults in malarial districts, this is only true of those born in the locality—natives coming from neighbouring non-malarial districts into the malarial region being liable to contract the disease. But of the existence of acquired immunity there can be no doubt, since a person who has previously suffered from malarial attacks may, while apparently in good health, harbour large numbers of parasites in the blood. It would appear that for the maintenance of immunity frequently repeated re-infection (superinfection) is required. At present, however, the facts available do not enable us to determine the relative parts played by the development of artificial immunity on the one hand, and the existence of a natural immunity on the other, in relation to insusceptibility to malaria.

Our knowledge regarding the relationship of blackwater fever to malaria is also in an unsatisfactory condition. Blackwater fever often occurs, especially in Europeans, in tropical countries where malaria exists. It is characterised by pyrexia, darkly-coloured urine—the colour being due to altered hæmoglobin pigment—delirium and collapse, frequently ending in coma and death. By some the condition has been looked on as a separate disease, by others as the terminal stage of a severe malaria. With regard to the former view, no special parasite has yet been definitely demonstrated. Blanchard and Lefrou have described spirochætes of the leptospira type in the blood in blackwater fever, but J. G. Thomson has suggested that these may be artificial appearances similar to the so-called "pseudospirochætes" so often observed in blood by the dark-ground illumination method. Stephens has summed up the evidence for the second view by saying that malaria, apart from the occurrence of blackwater fever, is a relatively non-fatal disease; that in the great majority of cases there is direct or indirect evidence of the subject of the condition having suffered from repeated attacks of malaria; that while in all cases there must be an agent at work causing hæmolysis, the evidence in some cases points to the possibility of that agent being quinine. Christophers and Bentley came to the conclusion that the essential

feature in blackwater fever is an extracellular destruction of red corpuscles in the blood plasma, a lysæmia as they call it, and that this is not directly due to parasitic, osmotic, or chemical actions, but to a specific hæmolysin arising in the body as the result of the repeated blood destruction. But the presence of an autolysin in the serum has not been established. It is noteworthy also that in attacks of blackwater fever the serum seldom shows any marked degree of staining with hæmoglobin. The occurrence of lysæmia may be precipitated by an acute attack of malaria especially when under certain circumstances this is associated with the administration of quinine. With regard to this view, however, it still remains to be determined what factors are responsible for the onset of an attack of blackwater fever. The evidence obtained by J. G. Thomson in recent investigations of the condition in Rhodesia has been strongly in support of the view that blackwater fever is the sequel of repeated attacks of malignant malaria.

Methods of Examination.—The parasites may be studied by examining the blood in the fresh condition, or by permanent preparations. In the former case, a slide and cover-glass having been thoroughly cleaned, a small drop of blood from the finger or lobe of the ear is caught by the cover-glass, and allowed to spread out between it and the slide. It ought to be of such a size that only a thin layer is formed. A ring of vaseline is placed round the edge of the cover-glass to prevent evaporation. For satisfactory examination an immersion lens is to be preferred. The amœboid movements are visible at the ordinary room temperature, though they are more active on a warm stage. With an Abbé condenser a small aperture of the diaphragm should be used.

For diagnosis, as well as for detailed study of the parasites, permanent preparations are required; these are best made by means of dried films, which are then fixed and stained by one of the Romanowsky methods, as described on p. 117. When such stains are not available, the dried films should be fixed by one of the methods described on p. 100, and then stained by methylene- or thionin-blue. In examining for the presence of malarial parasites it is most important that quinine should not have been administered before blood films are made. It must be remembered that the parasites may be very scanty in the blood, especially in early infections and in cases which have been treated with quinine. Accordingly, several hundred fields should always be examined.

Ross's "thick film process" may be used to aid the finding of scanty organisms. Here about as much blood as is used in a hæmoglobin determination (20 c.mm.) is taken on a slide, and, being spread out only so much as to occupy the area of an ordinary cover-glass, is allowed to dry. The hæmoglobin is removed by treating with distilled water, and the preparation is then fixed with methyl alcohol and stained by one of the Romanowsky methods; the parasites can then be readily found. Ross and Thomson have

modified the method for enumeration purposes. They take a definite small amount of blood, say 1 c.mm., and discharge it on a slide as one or more droplets, which are dried and treated as above. The whole blood is then carefully searched with an oil immersion lens with the aid of a movable stage, and the total number of parasites present is counted.

Amœbic Dysentery

In a previous chapter it has been pointed out that the term "dysentery" has been applied to conditions of different etiology, and the relations of bacteria as causal agents have been discussed (*vide* p. 429). We shall consider here that variety of tropical dysentery which is due to an amœba, and hence often known as *amœbic dysentery*.

Amongst the early researches on the relation of organisms to dysentery probably the most important are those of Lösch, who noted the presence and described the characters of amœbæ in the stools of a person suffering from the disease, and considered that they were probably the causal agents. Further observations on a more extended scale were made by Kartulis with confirmatory results, this observer finding the same organisms also in liver abscesses associated with dysentery. Councilman and Lafleur, working in Baltimore, showed that this variety of dysentery can be distinguished from other forms, not only by the presence of amœbæ, but also by its pathological anatomy. The intestinal lesions, to which reference is made below, are of a grave character, mortality is relatively high, and recovery, when it occurs, is protracted on account of the extensive tissue changes. The subject was, however, complicated by the fact that a somewhat similar organism—the *Amœba coli*—had been previously found in the intestine in normal conditions and in other diseases than dysentery (by Cunningham and Lewis and others), and additional research confirmed these results. The characters of the common amœba of the colon and an amœba of dysentery were carefully worked out by Schaudinn, who recognised them to be quite distinct species, and gave to them the names of *Entamœba coli* and *Entamœba histolytica* respectively. Huber afterwards described an entamœba of dysentery, which in the encysted stage contained four nuclei. Viereck confirmed these observations and gave the name *Entamœba tetragena* to the organism. This organism was shown to have pathogenic properties. Further research has resulted in its being generally recognised that *E. histolytica* and *E. tetragena* are the same. Schaudinn's name of *E. histolytica* has been retained, but its

process of encystment corresponds with that originally described in the case of *E. tetragena*. Moreover, a small entamœba, described by Elmassian under the name *E. minuta*, is also now known to represent merely a stage in the life-history of *E. histolytica*. Within recent years three other species of intestinal amœbæ have been identified. These, like the *E. coli*, are non-pathogenic commensals (p. 669).

Entamœba histolytica, as seen in the stools of acute dysentery, occurs in the form of rounded, oval, or pear-shaped cells, the rounded cells measuring 15–50 μ, usually about 30 μ, in diameter (Figs. 184–185, and Plate VI., Fig. 23). When at rest, a somewhat clear, highly refractile ectoplasm and a granular or sometimes

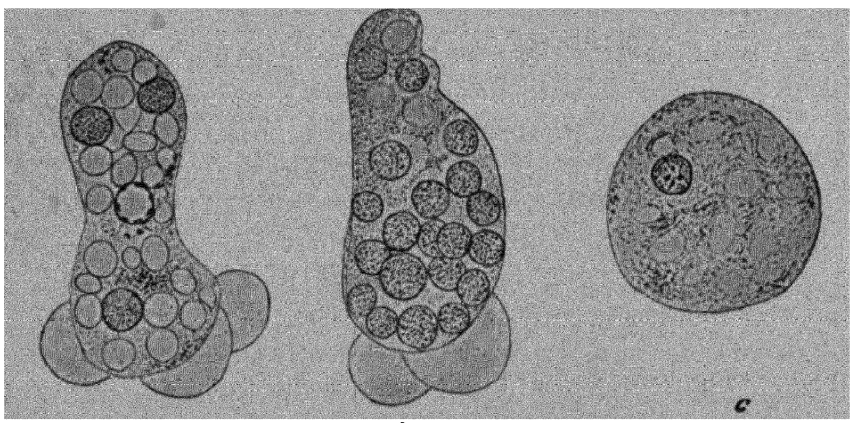


FIG. 184.—*Entamœba histolytica.*

a and *b*, amœbæ as seen in the fresh stools, showing blunt amœboid processes of ectoplasm. The endoplasm of *a* shows a nucleus, three red corpuscles, and numerous vacuoles; that of *b*, numerous red corpuscles and a few vacuoles.

c, an amœba as seen in a fixed film preparation, showing the small rounded nucleus (Kruse and Pasquale). × 600.

vacuolated endoplasm may sometimes be distinguished, though this is not always the case. The nucleus is rounded, as a rule, about 7 μ in diameter, and is seen with difficulty; its position is usually excentric, and is sometimes quite at the margin of the endoplasm. In stained specimens it is seen to be poor in chromatin, which is arranged as small granules under the nuclear membrane; a small chromatic karyosome is present in the centre of the nucleus (Fig. 185, A, B, C).

In fresh material many of the amœbæ show movement, throwing out and retracting blunt and well-defined pseudopodia of hyaline ectoplasm, which contrasts with the granular endoplasm. These appearances when present are characteristic and of great service in the identification of the organism. The

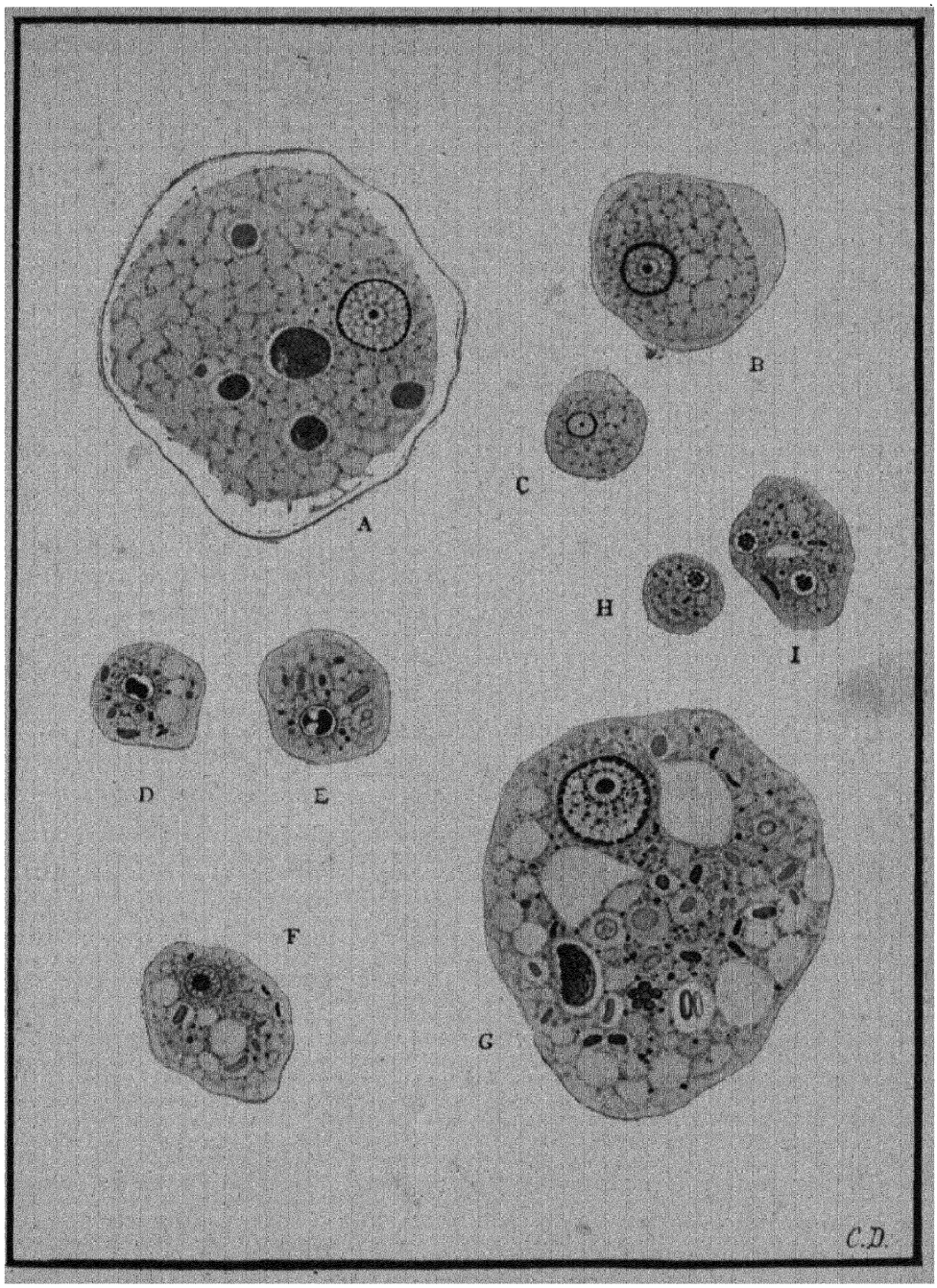


FIG. 185.[1]—Typical specimens of the intestinal amœbæ of man. From fixed and stained preparations. ×1500.

A, B, C. *Entamœba histolytica.* (A) Large tissue-inhabiting form, containing remains of red corpuscles: from stool in acute amœbic dysentery. (B) Precystic form, belonging to a race forming cysts usually 12 μ to 14 μ in diameter. (C) Precystic amœba of a race forming cysts usually 7–8 μ in diameter.

D, E. *Endolimax nana.*

F. *Iodamœba bütschlii.*

G. *Entamœba coli.*

H, I. *Dientamœba fragilis* uninucleate and typical binucleate individuals respectively.

[1] We are indebted to Colonel Byam and Dr. Archibald, and to Mr. Clifford Dobell for permission to use Figs. 185–186, which are reproduced from *The Practice of Medicine in the Tropics.*

amœboid movements are often of an active character, though they do not usually lead to much change in position. Red corpuscles, remains of cells, and occasionally bacteria may be present within the amœbæ. The ingestion of red corpuscles, though not always present, is a feature peculiar to the organism, and thus of special importance in its recognition; a considerable number of red corpuscles may be contained in an amœba. The amœbæ usually die and undergo disintegration in a comparatively short time after being removed from the body; the stools ought therefore to be examined in *as fresh a state as possible.* The appearances described are those found under ordinary con ditions of examination, but Dobell finds that when the amœbæ are quite fresh and healthy, they show active locomotion while endoplasm and ectoplasm are not clearly differentiated, and that there is little or no vacuolation present. He also considers that they do not ingest bacteria, and that the presence of bacteria within them indicates an invasion of degenerated amœbæ or, occasionally, a true parasitism by the bacteria.

Multiplication of the amœbæ occurs by division into two equal cells, but accounts given of the process vary. Appearances of division are rarely seen in the stools, and the process occurs mainly, if not exclusively, in the lesions. Dobell, from a study of sections of the lesions in the cat's intestine (*vide infra*), finds that when a cell is going to divide the chromatin increases in amount and becomes disposed in threads and granules, while the nucleus becomes spindle-shaped. It then undergoes constriction and division, each half assuming the circular form, and then division of the cell follows. He was unable to detect distinct chromosomes, and he regards the process as intermediate in character between mitotic and amitotic division.

As the symptoms of the disease abate, the entamœbæ undergo certain changes, which ultimately result in their encystment. The cysts, which are formed when the stools begin to regain their formed character, are spherical and relatively small, measuring 10–15 μ in diameter. (Dobell and Jepps have found that the average size varies in different strains.) The cyst wall is thin, with double contour, and within the cyst four, or sometimes only two, nuclei can be seen in the fresh condition. In fixed and stained specimens the nuclei are seen to have the chromatin at the periphery as in the active amœbæ, but are relatively richer in chromatin (Fig. 186, J–P). Beside the nuclei one or more elongated chromidial bodies may be seen, and there are also droplets containing glycogen. Such cysts may be found in the fæces for a long time after dysenteric

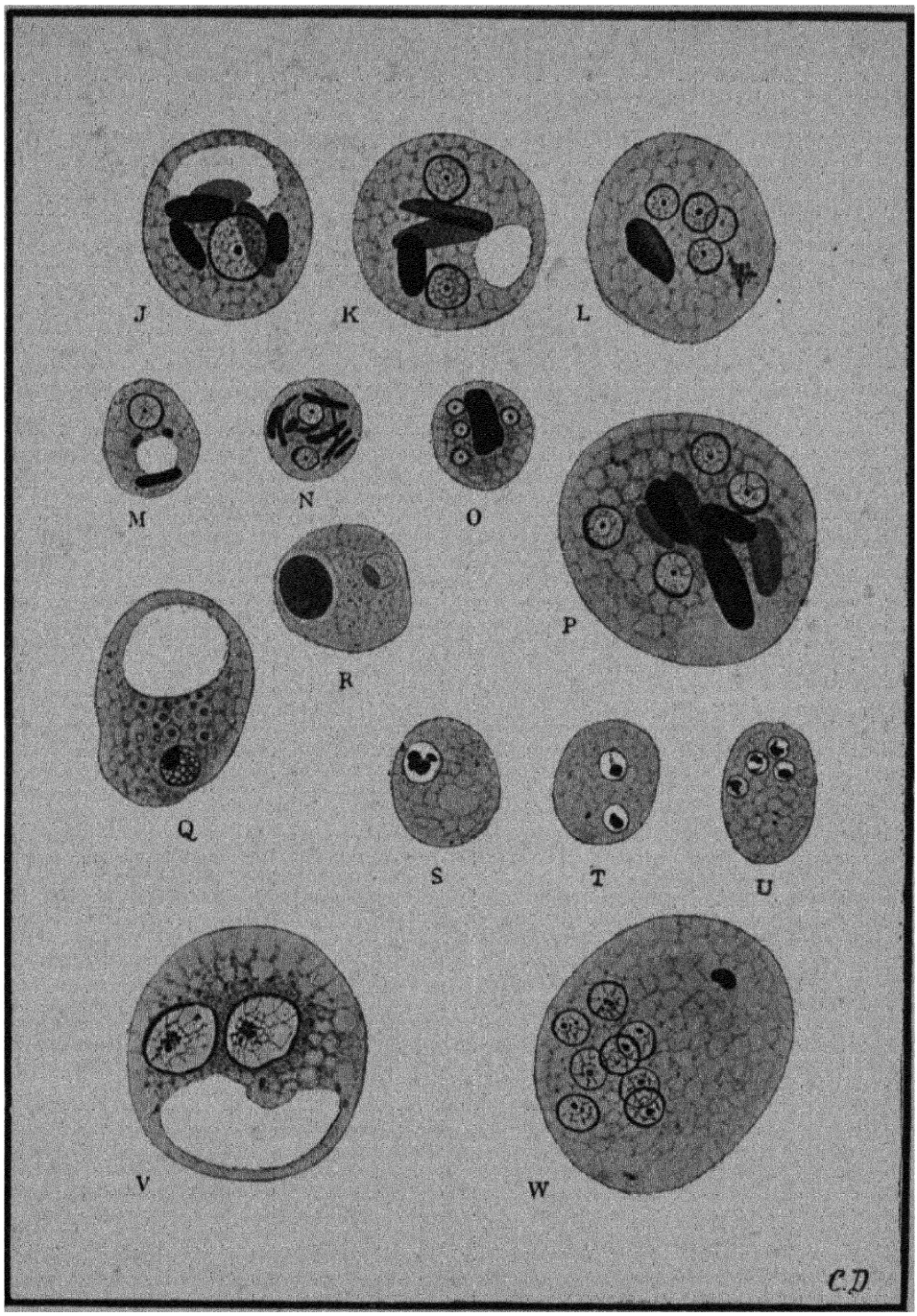


FIG. 186.—Typical specimens of the cysts of the intestinal amœbæ of man. From fixed and stained preparations. ×1500.

J–P. Cysts of *E. histolytica*. J, K, L, uninucleate, binucleate, and quadrinucleate specimens belonging to a strain forming cysts of medium size (mostly 12 μ to 13 μ in diameter); M, N, O, small cysts belonging to a strain with cysts averaging 6·6 μ in diameter; P, large cyst belonging to a strain with cysts of average size 15 μ.

Q, R. Cysts of *I. bütschlii*; R, stained with Best's carmine, showing glycogen mass.

S, T, U. *E. nana*—uninucleate, binucleate, and quadrinucleate cysts.

V, W. *E. coli*—binucleate and eight-nucleate cysts.

(The cyst-walls are not shown, as they are usually invisible in specimens mounted in balsam.)

symptoms have disappeared, and as they are the means of infecting other persons, the individuals passing them are to be regarded as carriers dangerous to the community; such individuals are known as "convalescent carriers." Cysts may be present also in the fæces of those who have never suffered from dysentery—"contact carriers"; in them infection with the entamœba has occurred, and though slight lesions are probably present, they are not sufficient to give rise to symptoms. The occurrence of liver abscess has been recorded in such carriers. In the transition from the active amœboid form to the cystic stage the following changes occur. The amœba becomes smaller and the nucleus more distinct and richer in chromatin, though still maintaining its characteristic features. Further diminution in size occurs, probably by division, the small cell loses its amœboid property, and a hyaline cyst-wall forms around it. The nucleus then divides into two or into four, as the case may be. These transition forms are to be met with in stools which are losing the typically dysenteric character. Unlike the free amœbæ, the cysts have considerable powers of resistance, and in the moist condition may survive for several weeks outside the body. They represent a resting and resistant stage of the parasite, by means of which fresh infection occurs (*vide* p. 673). It is important to recognise that they are not present in acute cases, and that accordingly infection is spread mainly, if not exclusively, by convalescents and carriers. They are found only in the intestine, and are never present in the secondary abscesses. The cysts remain in the fæces for a long time, and they have been found several years after dysenteric symptoms have disappeared. It is considered by some that they may persist during the rest of the patient's life. In connection with the treatment of amœbic dysentery by emetine, it is to be noted that failure to lead to cure in some cases cannot be ascribed to resistance to the drug on the part of cysts which may be present.

Non-Pathogenic Amœbæ.—As has been mentioned above, four other species of intestinal amœbæ are met with in man. These are *E. coli*, *E. nana*, *Iodamœba*, and *Dientamœba fragilis*. All of them are non-pathogenic commensals. The first mentioned belongs to the same genus as E. histolytica, each of the others to a separate genus. In distinguishing them, the characters of the nuclei and cysts are of most importance. The following is a short description of the four species:

The *Entamœba coli*, an intestinal commensal of common occurrence, is of about the same size as *E. histolytica*, but on the

whole is a little larger. When at rest it shows no differentiation into ectoplasm and endoplasm; the nucleus is readily seen, and shows a highly refractile membrane with chromatin lining it and in the interior The characters of the nucleus, as seen in fixed and stained preparations, are shown in Fig. 185, G. The protoplasm has a granular appearance, and in it there are often small vacuoles containing bacteria, food particles, etc.; glycogen also is present During amœboid movement, which is usually sluggish, some delicate processes of ectoplasm come into view, but the characteristic blunt protrusions described above in the case of the *E. histolytica* are not met with. Red corpuscles are rarely if ever found in the interior, but bacteria, food particles, etc., are often abundant (Fig. 185, G) The cellular changes in the encysting of the *E. coli* correspond with those of E. histolytica, and the ultimate result is the formation of a fairly large cyst (Fig. 186, V, W), which contains from two to eight nuclei. As seen in the fresh state in fæces, the cysts measure on an average 15–20 μ in diameter, the cyst-wall is distinct and refractile, the protoplasm is granular, and in the interior the nuclei are clearly visible. The nuclei have a similar structure to those of the free amœbæ. Glycogen is abundant in the cysts, but chromatoid bodies are usually absent. They are in these ways distinguishable from the cysts of E. histolytica

Endolimax nana (formerly called *Entamœba nana*). This is a small amœba about 8–12 μ in diameter and somewhat resembling a small *E. coli* in its appearance and movements. Like the latter, also, it often has in its protoplasm vacuoles containing ingested bacteria. In fixed and stained preparations its small nucleus is characterised by its relatively large karyosome, which varies much in shape and in its position within the nucleus (Fig. 185, D, E). The amœbæ are not infrequently parasitised by a small micro-organism belonging to genus Sphærita, the small oval and highly refractive spores of which form regular cluster-like masses of cocci (Dobell). The cysts of *E. nana* are of about the same size as the free forms, and when mature contain four minute nuclei, the karyosome in each having the peculiar character referred to (Fig. 186, S, T, U). They may, in addition, contain glycogen and small granules or rods. *E. nana* is a common commensal of the bowel, and has a wide geographical distribution; it has often been found in this country in those who have never been abroad. Though it has been met with most frequently in conditions of intestinal disorder, it also occurs in quite healthy individuals, and there is no evidence that it has any pathogenic properties. Thomson and Robertson have recently obtained cultures of *E. nana* in the medium of Boeck and Drbohlav (p. 670), in which it grows fairly well and presents the same features as in the fæces. No cysts were observed in the cultures.

Iodamœba bütschlii.—As seen in the fresh fæces, the organisms measure 8–12 μ in diameter and resemble somewhat small specimens of the E coli. As in the latter, there is little differentiation between ectoplasm and endoplasm, and within the endoplasm there are many vacuoles containing ingested bacteria, etc. The nucleus is vesicular and contains a large central karyosome (Fig. 185, F). The cysts are about the same size as the free forms. Each cyst contains a single nucleus in which a similar karyosome is situated

at the periphery, and usually also a comparatively large mass of glycogen (Fig. 186, Q, R). This species is of less common occurrence than the two previously described.

Dientamœba fragilis (Jepps and Dobell).—This organism, which is rare, is distinguished by usually possessing two nuclei of similar structure, though uninucleated forms also are met with. It is of small size, measuring 5–11 μ in diameter. Each nucleus (1·5–2 μ in diameter) is vesicular and contains a central karyosome of granular structure with a clear zone around it (Fig. 185, H, I). The cysts of this organism have not yet been found.

Cultivation.—Cultures of amœbæ in association with various bacteria were obtained by several workers, *e.g.* Lesage, Musgrave and Clegg, Noc, and others, from cases of dysentery and also from various sources outside the body, but in no instance was the identity of the amœba grown with the E. histolytica established; and the view was gradually gaining ground that the latter was a strict parasite, not cultivable outside the body. In 1925, however, Boeck and Drbohlav published an account of successful cultivation on a medium composed of Locke's solution, egg, and serum or albumen (*vide infra*). Growth was obtained by inoculating the medium with material from the stools of two cases of dysentery, and sub-cultures, made by transferring some fluid from the deep part of the culture tube by means of a glass pipette, were maintained through many generations—in the case of one strain for over eight months. The amœbæ in cultures were found to be comparatively short-lived, dying out after four or five days, and sub-cultures were made every two days. The formation of cysts in cultures was noted on only one occasion. On injecting kittens *per rectum* with the sub-cultures, Boeck and Drbohlav were able to produce typical dysenteric lesions, this result being obtained in eleven out of sixteen kittens used. In two of these, liver abscesses also developed. The inoculation experiments were carried on over a period of five months of sub-culturing, and there was no evidence that the amœbæ became diminished in virulence. These cultural results have been confirmed by Thomson and Robertson, and by Dobell and Laidlaw, who carried out a long series of sub-cultures, and observed also the formation of cysts from time to time. The latter observers found that the addition of sterile solid rice-starch to the medium of Boeck and Drbohlav gave more luxurious and prolonged growth of the amœbæ, and also enabled the whole life cycle, including excystation from the cysts, to be observed *in vitro*.

Medium of Boeck and Drbohlav.—Four eggs are washed, brushed with alcohol, and broken into a sterile flask containing glass beads;

50 c.c. of Locke's physiological solution are then added, and the mixture is broken up by shaking. Test tubes are filled with a sufficient quantity of the mixture to produce slants 1–1½ inches in length upon coagulation by heat. The tubes are slanted in an inspissator and heated at 70° C. until the mixture has solidified. They are then transferred to the autoclave and sterilised. The medium in each tube is covered to a depth of 1 c.c. above the slant with a mixture composed of eight parts of Locke's sterile solution and one part of sterile inactivated human blood serum. The tubes are then incubated to determine sterility. In place of the human serum a solution of crystallised egg albumen may be substituted. A 1 per cent. solution of the albumen in Locke's solution is prepared and sterilised by passing through a Berkefeld filter. It is then added to the tubes containing the egg slants as above described. The initial reaction of these media varied from Ph 7·2 to 7·8 and required no adjustment.

Distribution of E. histolytica.—As already stated, the organisms are usually found in large numbers in the contents of the large intestine in amœbic dysentery. Their real habitat, however, is the tissues, where they exert a well-marked action. The lesions are chiefly in the large intestine, especially in the rectum and at the flexures, though they may also be present in the lower part of the ileum. At first there are local swellings on the mucous surface, chiefly due to a sort of inflammatory gelatinous œdema with little leucocytic infiltration; soon, however, the mucous membrane becomes partially ulcerated, more or less extensive necrosis of the subjacent tissues occurs, and gangrenous sloughs result. The ulcers thus come to have irregular and overhanging margins, and the excavation below is often of wider extent than the aperture in the mucous membrane. The amœbæ are found in the mucous membrane when ulcers are being formed, but their most characteristic site is beyond the ulcerated area, where they may be seen penetrating

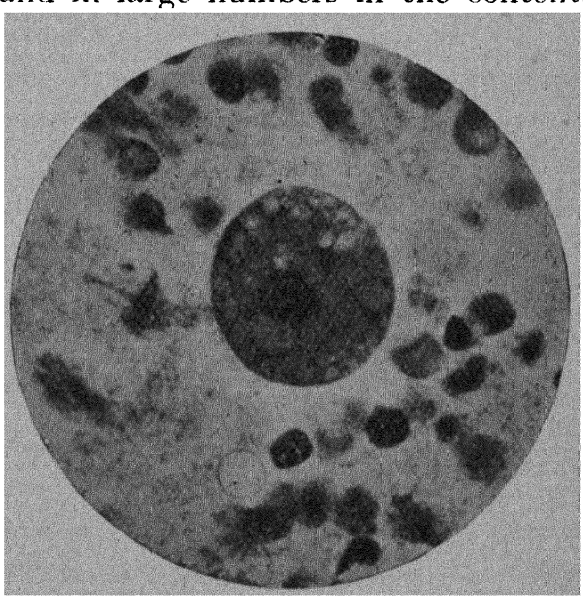


FIG. 187.—Section of wall of liver abscess showing an amœba of spherical form with vacuolated protoplasm. From a case published by Major D. G. Marshall. ×1000.

deeply into the submucous and even into the muscular coats. In these positions they may be unattended by any other organisms, and the tissues around them show œdematous swelling and more or less necrotic change, without much accompanying cellular reaction beyond a certain amount of swelling and proliferation of the connective-tissue cells. The amœbæ appear to produce a digestive softening of the tissues, hence the term "histolytica" is an appropriate one. These lesions are characteristic of amœbic dysentery.

As a complication of this form of dysentery, liver abscess is of comparatively common occurrence. It is usually single and of large size, but more than one may be present. The contents are usually a thick chocolate-coloured fluid of somewhat slimy consistence, which is largely composed of necrosed and liquefied tissue with admixture of blood in varying amount. In the abscess the amœbæ can be found, and usually are the only organisms present; occasionally secondary invasion by pyogenic organisms occurs. The amœbæ are most numerous at the spreading margin, and this probably explains a fact pointed out by Manson, that examination of the contents first removed may give a negative result, while they may be detected in the discharge a day or two later. The action here on the tissues is of an analogous nature, namely, a necrosis with softening and partial liquefaction, attended by little or no suppurative change. There is, however, evidence that the amœbæ may infect the liver without causing actual abscess formation, merely a hepatitis, and that this may be followed by cirrhosis. Abscesses are also met with in the lungs, and in such cases the amœbæ have been found in the sputum; as also when a liver abscess has ruptured into the lung, which not very infrequently happens. There have also been recorded a considerable number of cases of cerebral abscess in which the amœbæ have been found; most of these have been secondary to lung infection.

Experimental Inoculation.—Dysentery occurs occasionally in animals, *e.g.* in monkeys, but it is of comparatively rare occurrence. The disease sometimes results in the dog by experimental inoculation with dysenteric material, as was shown first by Lösch and afterwards by Kartulis, the latter producing the disease in one instance by the contents of a tropical abscess. Cats are, however, found to be more susceptible, especially young animals, and have been mainly used in investigation. Dysentery may be readily produced in them by introducing into the rectum mucus from a case of dysentery; and a similar result has been obtained by means of material from a liver

abscess containing amœbæ (Kruse and Pasquale). Infection may take place also by oral administration, but in this case the presence of cysts in the material is essential since the amœbæ are destroyed in the stomach, as was first shown by Quincke and Roos. The experimentally produced disease in cats is of an acute character and is usually fatal; in conformity with this, the cystic stage of the organism is not met with in the stools (*vide supra*). The most important experiments, however, were those carried out by Walker and Sellards on the human subject. They administered to Filipinos, who acted as volunteers, various amœbæ and entamœbæ or their cysts, the material being mixed with magnesium oxide or starch, and enclosed in gelatine capsules. The following were the results with the *E. histolytica*. Of twenty volunteers, eighteen were fed with the cysts, two being kept as controls. The result was that seventeen became parasitised after one feeding and one after three feedings, the cysts persisting in the stools; of these, four contracted dysentery, the average period of incubation being sixty days. In the case of the other amœbæ cultivable at that time, they found that though the organisms might be detected in the fæces after feeding with them, none of them became parasites and no pathogenic effects were produced. These results are of great importance both in demonstrating the specific pathogenic properties of the *E. histolytica*, and also in showing that it may become an intestinal parasite without causing dysenteric symptoms and lesions. Walker and Sellards concluded that the *E. histolytica* is a strict parasite, and that the source of infection is always another individual harbouring the organism in the intestine, and this view has received general support. In connection with this it may be noted that Wenyon and O'Connor examined the stools in nearly two thousand healthy individuals in Egypt and found that there was infection by *E. histolytica* in fully 5 per cent., whilst in less than a sixth of the infected individuals was there a history that they had suffered from dysentery. Observations made in more recent times show that *E. histolytica* has a much more widespread distribution than was formerly supposed. Yorke, Malins Smith, and others, for example, have found that the cysts are not infrequently to be found in various classes of the population in this country, the largest percentage of positives being among asylum inmates.

In the case of the *E. coli*, Walker and Sellards were able to bring about parasitism by feeding with the cysts of the organism, but no pathogenic effects followed. Their results accordingly

confirm the view previously held that it is a harmless organism. It has practically a world-wide distribution, and in certain countries is very common. Schaudinn found that in East Prussia as many as 50 per cent. of the population were infected with it, and confirmatory results with regard to its common occurrence were obtained by Craig in San Francisco.

Methods of Examination.—The fæces in a suspected case of acute dysentery ought to be examined microscopically *as soon as possible after being passed*, as the entamœbæ disappear rapidly, especially when the reaction becomes acid A drop is placed on a slide without the addition of any reagent, a cover-glass is placed over it but not pressed down, and the preparation is examined in the ordinary way or on a hot stage, preferably by the latter method, as the movements of the entamœbæ become more active. The addition of a solution of neutral red, 1 : 5000, is recommended by some, as it stains the entamœbæ a pale pink colour. In the examination for cysts, when the fæces have more of a formed character, a small portion of fæces is emulsified in saline or in Lugol's iodine solution, which brings out the nuclei rather more distinctly and stains glycogen granules. The cysts may be conveniently picked out by means of a dry lens and then examined under the oil immersion. In this case immediate examination of the fæces after being passed is not essential, as the cysts persist unchanged for several days.

For permanent preparations dried films are not suitable, as in the preparation of these the entamœbæ become distorted. Wet films should be used, and a very suitable fixing agent is composed of 2 parts of a saturated solution of corrosive sublimate in normal salt solution and 1 part of absolute alcohol ; they are then treated as already described (p. 100). For such films Heidenhain's iron hæmatoxylin has been found to be one of the best stains, but ordinary hæmalum gives quite good results.

In sections of tissue the entamœbæ may be stained by methylene-blue, by safranin, by hæmatoxylin and eosin, iron hæmatoxylin, etc. Benda's method of staining with safranin and light-green is also a very suitable one. Sections are stained for about an hour in a saturated solution of safranin in aniline oil water (p. 105), they are then washed in water and decolorised in a ½ per cent. solution of light-green in alcohol till most of the safranin is discharged, the nuclei, however, remaining deeply stained. In this method the nuclei of the entamœbæ are coloured red (like those of the tissue cells), the protoplasm being of a purplish tint.

Addendum—Entamœba gingivalis

The occurrence of amœbæ in the mouth has been recognised for a considerable time, but special attention has been directed to them more recently, as the view has been put forward that they are the cause of pyorrhœa. The amœbæ, so far as is known, all belong to the one species—*E. gingivalis* (it has also been called *E. buccalis*). The organism is a small amœba which tends to abound in certain morbid conditions of the mouth. It is of smaller

size than the *E. coli*, measuring about 10–20 μ in diameter, while its nucleus, seen with some difficulty in the living amœba like that of *E. histolytica*, is relatively small, measuring 3–4 μ. The amœbæ show active amœboid movements, throwing out rounded pseudopodia, and ectoplasm and endoplasm are clearly distinguishable. The endoplasm is granular, and often contains numerous rounded bodies or inclusions, which stain deeply with nuclear dyes, and have been supposed by some to represent the remains of nuclei of leucocytes or other cells. No cysts of this organism have been observed. Although the amœbæ have been observed in a fair proportion of healthy individuals, there is no doubt that they are specially abundant in morbid states. They are usually present in pyorrhœa, and are commonly met with in dental caries, spongy gums, and in the tartar of the teeth. It has not been established that the organism is responsible for pyorrhœa or any other morbid change, and the evidence goes to show that it is a fairly common commensal of the mouth, which becomes increased in pathological conditions.

CHAPTER XXVIII

PROTOZOAL DISEASES (*continued*): TRYPANOSOMIASIS, LEISHMANIASIS, ETC.

The Pathogenic Trypanosomes

The trypanosomata are protozoal organisms belonging to the sub-class Flagellata, and many members of the genus have come to be recognised as living in the blood and tissues in various animals, and as causing important disease conditions. These diseases present many general resemblances to one another. They tend to be characterised by wasting, cachexia, anæmia, fever, often of an intermittent type, and irregular œdemas, and frequently have a fatal result. In many cases the infective agent has been proved to be conveyed from a diseased to a healthy animal by the agency of blood-sucking insects. In the human subject two species of trypanosomes are met with, *T. gambiense* and *T. rhodesiense*.

Morphology and Biology of the Trypanosomes.—If a drop of fresh blood containing trypanosomes be examined between a slide and coverslip, the organisms are seen to be fusiform, one end passing into a pointed flagellum. In the living condition the trypanosomes are usually actively motile by an undulatory movement of their protoplasm and a lashing of the flagellum, but many species show little tendency to movements of progression. From the fact that in progression the flagellum is in front, the end at which it emerges is regarded as anterior. The size varies, but many, including those parasitic in man, are about 13–33 μ long and about 1·5 to 3 μ broad. The method of examining the fresh blood is the one most likely to reveal the presence of trypanosomes, if these are present in small numbers, since they are readily detected by the movement which they impart to the red corpuscles round about. But the minuter structure of the organisms can be studied in preparations fixed after drying or preferably when wet, and stained by Romanowsky dyes, such as those of Leishman or Giemsa.

For staining trypanosomes (or Leishman-Donovan bodies) *in sections* so as to bring out the chromatin structures, Leishman

recommended the following method : Sections of 5 μ thickness are made and carefully fixed on slides. The paraffin is very thoroughly removed, and after the section has been brought into distilled water, the excess of the latter is blotted off. A drop of fresh blood serum is then placed on the preparation and allowed to soak in for five minutes. The excess is removed by blotting, and the remainder is allowed to dry on the section, which is now treated with a mixture of two parts of Leishman's stain and three of distilled water, and placed in a Petri dish for 1 to 1½ hours. The preparation is very deeply stained, the nuclei being almost black, and decolorisation and differentiation are effected by alternately applying the acetic acid and caustic soda solutions (commencing with the acid) used in the application of the stain to ordinary histological sections (*vide* p. 118), the effects being carefully watched with a low power. The essential part of the method is the application of the blood serum ; Leishman suggested that its effect is to restore the alkalinity of the tissue.

In preparations stained by the above methods the protoplasm of trypanosomes stains blue, and in certain species some parts are more intensely coloured than others. Sometimes it contains violet-coloured granules (chromatin granules), and occasionally there appears in it slight longitudinal striation. Two bodies are always present in the protoplasm. An oval granular body staining purple, the trophonucleus or macronucleus, is usually near the middle, and towards the posterior end is a minute intensely stained purple granule known as the kinetonucleus, kinetoplast, or micronucleus, which may be differentiated into the blepharoplast (the granule from which the axoneme arises —*vide infra*), and deeply staining material, the parabasal body (the micronucleus was held by Laveran to represent the centrosome from the analogy of appearances in certain spermatozoa which closely resemble trypanosomes in structure). The micronucleus is often surrounded by an unstained halo, and in its neighbourhood, in certain species, a vacuole has been described. From the micronucleus or from its neighbourhood there arises an important structure in the trypanosome—the undulatory membrane. This is of varying breadth, has a sharp undulating free margin, and surmounts the protoplasm of the organism like a cock's comb ; it narrows towards the anterior end, where it becomes the flagellum. A filament (axoneme) which stains of the red tint of chromatin, commences at the blepharoplast and runs along the free margin of the undulating membrane ; it then forms the core of the flagellum. The latter is continuous with the protoplasm of the body of the organism. Motion is chiefly effected by the undulations of the membrane and of the flagellum. In different species of trypanosomes variations occur in shape,

in length, in breadth, in the position of the micronucleus (and therefore in the length of the undulating membrane), in the breadth of the membrane, in the length of the free part of the flagellum, in the shape of the posterior end, which is sometimes blunt, sometimes sharp, and in the presence or absence of free chromatin granules in the protoplasm. It may be said that the differentiation of species of trypanosomes is often a task of great difficulty, as both morphological and experimental study is necessary.

Multiplication in the body fluids ordinarily occurs by longitudinal, amitotic division, the parasite having increased in length and breadth beforehand. First of all, the micronucleus divides, sometimes transversely, sometimes longitudinally, then the macronucleus and undulating membrane, and lastly the protoplasm. In some species only the root of the flagellum divides, so that in the young trypanosomes the flagellum is short and subsequently increases in length (T. lewisi). It has been held that the whole flagellum takes part in the general splitting of the organism ; but, according to Wenyon, after the blepharoplast has divided, the original axoneme remains attached to one moiety and a new axoneme grows out of the other. The view has been advanced that in certain cases reproduction occurs by the formation of " latent bodies " consisting of the nucleus fused with the blepharoplast (Moore and Breinl), or by the endogenous formation in the nucleus of " chromidial buds " (Minchin), or " infective granules " (Henry and other observers), which when extruded from the protoplasm develop into trypanosomes ; but according to other workers these appearances may be due to degenerative changes.

In many species of trypanosomes the parasites in the circulating blood show differences in shape and size. There is a form long and slender in both body and nucleus, the free part of the flagellum being longer than the body and the protoplasm devoid of granules. At the same time a broader form of the organism with a larger and rounder nucleus and a blunter posterior extremity is found ; its undulating membrane is narrow and the free part of the flagellum is shorter than the body, and the protoplasm contains granules. According to one view, this polymorphism indicates sexual differentiation, the former being the male form and the latter the female, but intermediate forms are also met with and conjugation has never been observed. Accordingly, it is probable that the variations indicate merely different stages of growth. Whether any pathogenic significance is to be attached to the occurrence of these different forms

is at present unknown. Further, in chronic infections the number of organisms present in the peripheral blood varies, and thus the potentiality of infection by means of an invertebrate carrier also varies. When the organisms are absent from the blood they may still be found in the solid organs and in the bone marrow, and in such situations may go through a phase of development. In T. cruzi such a stage has been demonstrated in tissue cells.

The outstanding fact in the biology of the pathogenic trypanosomes is that infection from vertebrate to vertebrate takes place through the agency of biting or blood-sucking insects, or, in the case of frogs and fishes, by leeches. The mere mechanical transference by such invertebrates is possible, and in certain cases multiplication of the organisms in the biting apparatus of the invertebrate occurs. Such a mechanical or semi-mechanical transference, however, may at times play only a subsidiary part in spreading the infection, for in many cases a considerable period may elapse before an insect which has ingested infected blood becomes infective for new hosts. Here the parasite undoubtedly goes through a cycle of development within the invertebrate, the details of which are in some instances as yet undetermined. In the alimentary tract of the insect, the trypanosomes are seen to undergo modifications in form. They may show simple division, by which the resulting individuals become smaller; the relation of kinetonucleus and trophonucleus may be altered, the former coming to lie anterior to the latter, while the undulating membrane and flagellum become rudimentary (crithidial forms). In other cases, organisms resembling Leishmaniæ result. Koch and Kleine also found in the intestine agglomerations of immature forms which they ascribed to the results of sexual conjugation. The behaviour of the organisms in the invertebrate host before they again become infective for vertebrates varies in different instances. Thus T. gambiense passes finally to the salivary glands of the tsetse fly; on the other hand, T. lewisi travels to the rectum of the rat flea and the infective forms pass out in the fæces. While the analogy of what happens in the malarial parasite suggests the possibility of a sexual element in a trypanosomal cycle, there is at present no definite proof that such a stage has ever been observed.

It has been found possible to cultivate a number of the trypanosomes outside the bodies of their natural hosts, the first work having been done by Novy and MacNeal, who introduced a special medium for the purpose.

Nicolle's modification of Novy and MacNeal's blood agar ("N.N.N. medium") for the cultivation of trypanosomes, leishmaniæ, etc., is prepared as follows: Mix thoroughly in a flask 14 grams agar, 6 grams NaCl, and 900 c.c. water, and steam for two hours (the medium is not neutralised or cleared). Filter through cotton wool and tube 2 c.c. in tubes of ½-inch diameter; autoclave at 120° C. for twenty minutes. Cool the medium to 50° C. in a water bath; then into each tube introduce 20 drops of whole rabbit's blood obtained with aseptic precautions as described on p. 133. Mix thoroughly by "rolling" in the hand, and allow the medium to solidify in the sloped position. Incubate the tubes for several days at 37° C. to test sterility. As the presence of abundance of condensation water is essential, evaporation must be prevented by capping the tubes. Inoculation is made by introducing the infective material by means of a capillary pipette into the water of condensation.

In cultures, the organisms may divide longitudinally, as seen in the blood, or crithidial or leishmania forms may result, the former being often arranged in rosettes containing a large number of individuals with their flagella pointing in one direction. A fresh infection may sometimes be originated by introducing such cultures into suitable animals.

While some trypanosomes give rise to serious disease, in the case of others a heavy infection may occur without the animal suffering any apparent inconvenience, and a form producing disease in one species may be present in considerable numbers in another species without causing any pathogenic effects.

Sleeping Sickness.—Since the year 1800 the disease called sleeping sickness, sleeping dropsy, or negro lethargy has been recognised as prevailing on the West Coast of Africa from the Senegal to Lagos, and in the parts lying behind the coast between these regions. It has also been found to be rife from Cameroon to Angola and in the Congo valley, and to a less extent up the Niger and its tributaries. In 1901 it began to appear in the Uganda Protectorate, where it has wrought very serious havoc amongst the native population, and the investigations carried on in that region have led to a knowledge of its cause. The disease is characterised in the early stages by a change in disposition leading to moroseness, apathy, disinclination for work or exertion, and slowness of speech and gait. There may be headache, indefinite pains about the body, the evening temperature may be elevated several degrees, the pulse tends to be soft and rapid, and in a very large number of cases the superficial glands of the body are enlarged. In a rapid case the lethargy becomes more pronounced; fine tremors,

especially of the tongue and arms, develop ; progressive emaciation occurs ; blood changes appear, consisting of a progressive diminution of the red cells and of the hæmoglobin, and of a lymphocytosis in which the percentage of both the large and small mononuclear cells is increased, so that the former may constitute from 20 to 30 and the latter from 30 to 40 per cent. of all the white cells present. As the disease progresses the drowsiness increases till it deepens into a coma from which the individual cannot be roused. Often during the disease there occur irregular œdematous patches on the skin, and sometimes erythematous eruptions, and effusions into the serous cavities. Not every case runs a progressively advancing course. Sometimes along with enlargement of glands the chief early feature is the occurrence from time to time of attacks of fever which may be mistaken for malaria, and from these apparently complete recovery may take place ; recurrence, however, follows as a rule, and ultimately the typical terminal phenomena may commence. Such cases may go on for years, and it is probable that many patients die of pneumonia without exhibiting typical manifestations of the malady from which they really suffer. The disease is an extremely fatal condition, and probably no case where the actual lethargy is developed ever recovers.

As described by Mott, the most striking anatomical change is the presence of a chronic meningo-encephalitis and meningomyelitis. The pia-arachnoid is sometimes opaque and slightly thickened and may be adherent to the brain, and its vessels usually show some congestion. The sub-arachnoid fluid is sometimes in excess and occasionally may even be purulent. The membranes of the spinal cord show similar changes. The chief other feature is the presence of enlarged lymphatic glands in the body, but otherwise there is nothing special to note. With regard to the microscopic changes, the chief feature is a proliferation and overgrowth of the neuroglia cells, especially of those which are related to the sub-arachnoid space and the perivascular lymph spaces, with accumulation and probably proliferation of lymphocytes in the meshwork. The changes in the lymph glands are of similar nature, and resemble the infiltration of the perivascular lymphatics of the central nervous system. These changes are specially significant in view of the lymphocytosis present in the blood, which has already been noted, and which so often occurs in protozoal infections. In the nerve elements there are merely some atrophy of the dendrons of the nerve cells, a diminution of Nissl's granules, and an excentric position of the nuclei.

Trypanosoma gambiense.—The first case in which trypanosomes were found in the blood was recorded by Dutton in 1901 ; the patient, who was not obviously suffering from sleeping sickness, was a European then living at Bathurst on the Gambia. The progress of the disease was very slow, and was characterised by general wasting and weakness, irregular rises of temperature, local œdemas, congested areas of the skin, enlargement of spleen, and increased frequency of pulse and respiration ; death occurred a year after the case came under observation after an access of fever, and a striking fact was the absence of any gross lesion. During the time the patient was under observation trypanosomes were repeatedly demonstrated in the peripheral blood, and they also developed in monkeys and white rats inoculated with the blood. Pursuing further inquiries, Dutton and Todd demonstrated similar parasites in other Europeans and in several natives in the Gambia region, whilst about the same time Manson reported a case of the same kind from the Congo. It thus came to be recognised that in man there occurred a disease somewhat resembling nagana, in which trypanosomes could be demonstrated in the blood, and this was usually referred to as human trypanosomiasis, or trypanosome fever—the trypanosome being named the T. gambiense. Since T. gambiense usually occurs only in small numbers in the blood in man, its microscopic characters have been studied mainly in infections of animals such as guinea-pigs or rats. In these it measures on the average 15–30 μ, the nucleus is central and the blepharoplast close to the posterior end. The trypanosome is polymorphic, long slender forms with flagella and short broad forms without flagella, as well as forms of intermediate length, being found (Fig. 188 and Fig. 189, 1–5).

Owing to the seriousness of the epidemic of sleeping sickness in Uganda, a Commission of the Royal Society was dispatched in 1902 to investigate the condition. Castellani found in some cases in the cerebro-spinal fluid, especially when this was centrifuged, living trypanosomes resembling the T. gambiense, which seem at first to have been regarded as accidental. Bruce, pursuing the work of the Commission with Nabarro and Greig in 1903, made a series of examinations in several infected localities, and demonstrated the trypanosome in every case of the disease. This work formed the starting-point for inquiries, which proved that the parasite is the causal agent of the condition. The organisms were not seen in the cerebro-spinal fluid of patients dying of other diseases in the sleeping sickness area. On the other hand, it was found that if cerebro-spinal

fluid withdrawn from cases of the disease was injected into monkeys (especially *Macacus rhesus*), trypanosomes appeared in the blood, and in many cases in three or four months the animals died of an illness indistinguishable from sleeping sickness, and with the parasites in the central nervous system. Thus when T. gambiense is inoculated into monkeys they often contract a disease which ultimately presents the features of typical sleeping sickness. In inoculation of other species of

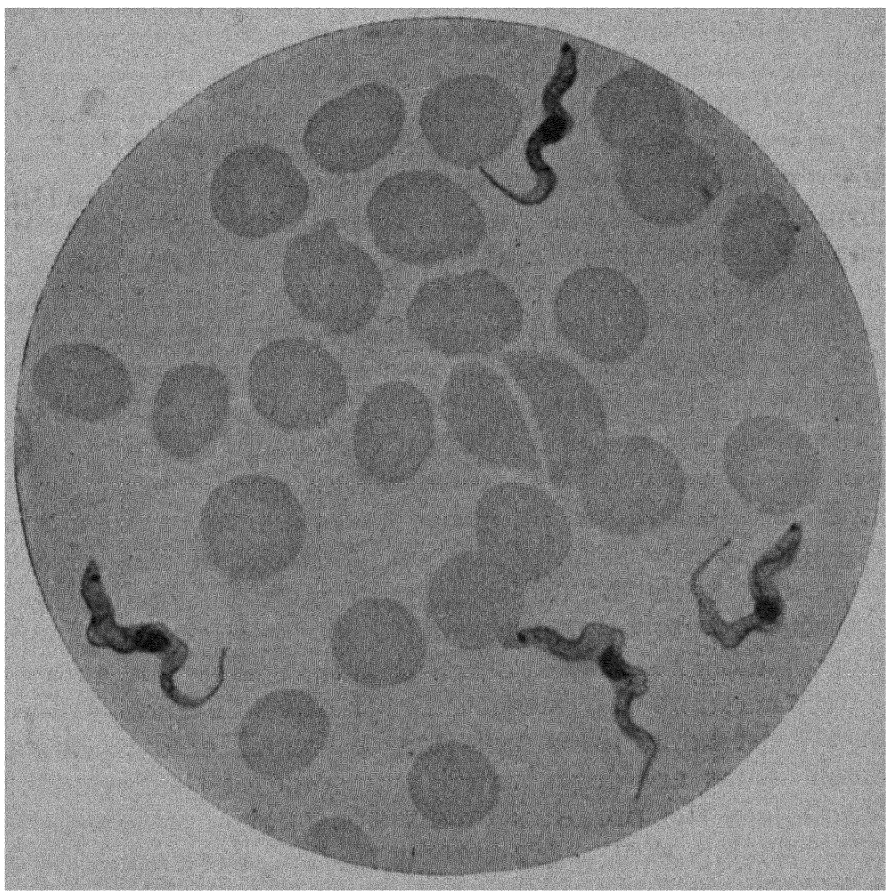


FIG. 188.—Trypanosoma gambiense from blood of guinea-pig. ×1000. See also Plate VI., Fig. 25.

animals, *e.g.* herbivora or the guinea-pig, in nearly every case a proliferation of the parasite takes place, as indicated by its appearing in the blood; but often either no disease occurs or this runs a very chronic course. Small animals such as guinea-pigs, rats, and mice can also be infected, *e.g.* by subcutaneous inoculation. In the latter the virulence can be increased by repeated passage through the same species, and the infection in rats or mice assumes a septicæmic character, but it never becomes so virulent for these animals as T. brucei. Under ordinary

conditions the trypanosomes do not long survive removal from the body.

It was found that in the parts round the north end of Victoria Nyanza where sleeping sickness was prevalent, the distribution of the disease exactly corresponded with the distribution of a blood-sucking insect, the *Glossina palpalis*, a species closely allied to the *Glossina morsitans* of nagana. When one of these flies was fed on a sleeping sickness patient and then allowed to bite a monkey, trypanosomes frequently appeared in the animal's blood, and the same result often followed when fresh flies caught in the sleeping sickness area were placed on a monkey. Kleine established the important fact that when Gl. morsitans was allowed to bite an animal suffering from nagana it did not become infective for about twenty days. This was confirmed for Gl. palpalis, in the case of monkeys infected with T. gambiense, by Bruce and those associated with him. Here it was found that infectivity did not appear till about thirty-two days after the fly had fed, and continued until at least seventy-five days. It was at first supposed that monkeys could not be infected with the trypanosomes from the bruised-up bodies of the fly, but Bruce succeeded in originating an infection with this material, results being positive during the first two days after the fly had bitten and then being negative till after the twenty-second day. Bruce noted that the renewed infectivity corresponded with the appearance of perfect trypanosomes in the salivary gland of the glossina. The cycle of development of the trypanosome in Gl. palpalis has been found by Bruce and his co-workers and by Robertson to be as follows. Thirty-six to forty-eight hours after ingestion of the infected blood many of the parasites are degenerated, but some of the broad forms are dividing; the products of division show crithidial [1] forms only for a short time. At the tenth day numerous trypanosomes of very variable shape and size are present; later, slender forms appear in increasing numbers, and these pass to the proventriculus and hypopharynx. They travel along the duct to the salivary gland and here crithidial forms develop; the latter again produce trypanosomes, and the appearance of these "metacyclic" forms about the twentieth day coincides with the infectivity of the fly for the vertebrate host. Only a small proportion of flies which have ingested trypanosomes in the blood of a patient become infective subsequently. The temperature has an important influence on the development of the trypano-

[1] In the crithidia the blepharoplast is situated anteriorly and the undulating membrane is only slightly developed.

somes in the fly; thus Kinghorn and Yorke found in the case of T. rhodesiense that under 24° C. the trypanosomes did not invade the salivary glands of Glossina morsitans, and hence the fly did not become infective. Certain facts having a serious bearing on the continued infectivity of a locality have emerged. It was found that a certain island on Lake Victoria Nyanza, which had been cleared of infective natives two years previously, still harboured infective flies. To account for this it must be supposed either that the glossina has an extended duration of life, or that the trypanosome exists among the wild animals. It has been found that cattle and wild herbivora can be infected with the parasite, and can through the medium of the fly infect monkeys. It is possible that such animals, while not suffering in any serious way themselves, are the means of maintaining infectivity. There is no definite evidence that, as Koch supposed, the crocodile harbours the trypanosome.

Early in the Uganda investigations the question arose as to whether the trypanosome of sleeping sickness was different from that discovered by Todd. This was forced on the inquirers by the fact that a large proportion of the natives in the sleeping sickness area were found to harbour trypanosomes in their blood, although not apparently suffering from the disease. Several cases were carefully examined in which trypanosomes were constantly present in the blood, but in which the patients from time to time suffered from fever, and during these pyrexial periods trypanosomes were found in the cerebro-spinal fluid. It was suggested that these cases were on the way to develop sleeping sickness. A very important observation was that while in sleeping sickness areas a large proportion of the native population harboured trypanosomes, this was not the case where sleeping sickness did not occur. Further, it was found that trypanosomes from the cerebro-spinal fluid of sleeping sickness cases and from the blood of persons harbouring trypanosomes, but not suffering from disease symptoms, gave rise in monkeys to the same group of chronic effects which resembled the last stages of the disease in man. These facts led the Commissioners to incline to the idea that trypanosome fever and sleeping sickness are due to the same cause, and represent different stages of the same disease. It has already been pointed out that a fatal termination can occur in trypanosome fever by an acute febrile attack or from intercurrent disease, and thus the terminal lethargic stage may only develop in a certain proportion of cases. Continued observation of prolonged cases of trypanosome fever, both in Uganda by Greig and Gray, and in this country

by Manson, has shown that sometimes the termination of a case is by the onset of typical sleeping sickness. There is now practically no doubt that the two conditions are etiologically identical. It is generally agreed, also, that there are no morphological differences between the trypanosomes from the two types of case : hence the name T. ugandense originally applied to the organism recovered from actual cases of sleeping sickness is no longer used.

The prevalence of trypanosomes in the blood of apparently healthy natives has raised the question of the possibility of tolerance existing and of immunity being established. It is possible that both phenomena occur, that not every infection results in multiplication of the parasite in the body of the victim, and that in certain cases where multiplication does occur, a resistance is developed which enables the body to kill the parasites. It has been suggested that, when this resistance is weak, the organism gains entrance to the cerebro-spinal system, and that then sleeping sickness results.

Trypanosoma rhodesiense.—In 1910, Stephens and Fantham observed certain peculiarities in the trypanosomes derived from a case of human trypanosomiasis occurring in an individual who had returned to England from Rhodesia. The organisms, as seen in the blood of small animals (rats, mice, and guinea-pigs), frequently presented a very blunt posterior extremity and the trophonucleus tended to approach the kinetonucleus and in certain cases to lie behind it (Fig. 189, 6–11); otherwise the trypanosomes closely resembled T. gambiense. Another feature of the case was that only Gl. morsitans, which up till then had not been suspected of being capable of transmitting trypanosomiasis to man, prevailed in the regions through which the patient had travelled. Shortly thereafter a serious outbreak of trypanosomiasis was reported from the country west of Lake Nyassa, and it is now known that the disease prevails on several of the northern tributaries of the Zambesi, in the adjacent parts of the Belgian Congo, and even in Portuguese East Africa in districts where only Gl. morsitans and not Gl. palpalis prevails. It was, however, shown by Kinghorn and Yorke, working on the Luangwa (a tributary of the Zambesi), that Gl. morsitans could transmit trypanosomes from human cases to rats, the cycle in the fly being at least eleven days, and that a definite percentage of wild flies in this region harboured the human parasite. There is thus no doubt that man, in widely extended regions of southern Central Africa, is exposed to danger when bitten by Gl. morsitans. Further, the opinion is

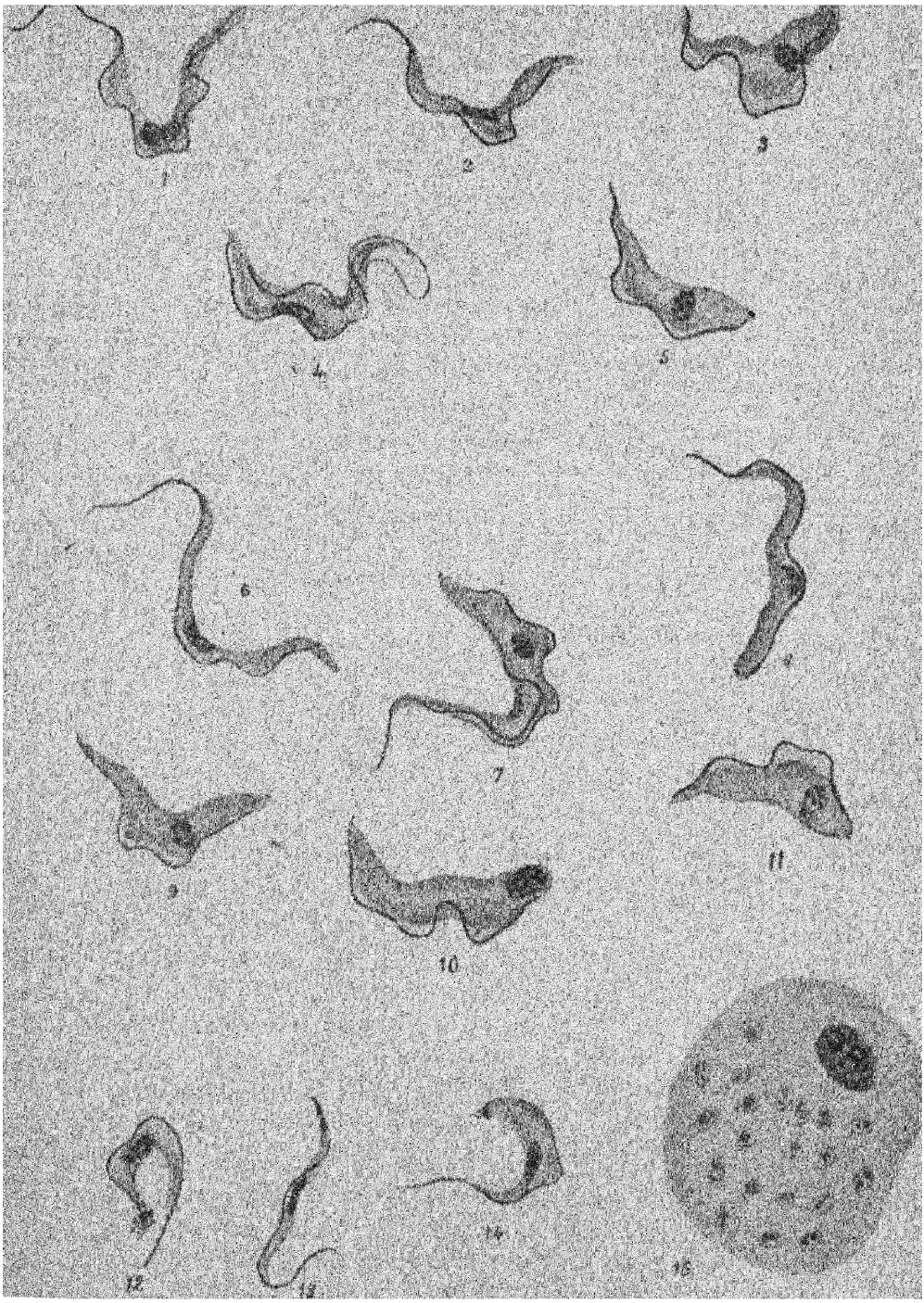


FIG. 189.[1]—Pathogenic Trypanosomes. × 1500.

1–5. T. gambiense.
6–11. T. rhodesiense.
12–15. T. cruzi.

[1] For this figure, reproduced from *The Practice of Medicine in the Tropics*, we are indebted to Colonel Byam and Dr. Archibald and to Professor Warrington Yorke.

generally accepted that T. rhodesiense is a species distinct from T. gambiense. The disease in man tends to be more acute; there is frequently not a terminal sleeping sickness stage, and there is less pronounced infection of lymphatic glands. The organism is also more virulent for animals, the duration of the illness being shorter, and the susceptibility of the sheep and goat is greater than towards T. gambiense. In both of these animals widespread œdema, especially of the face, is a marked characteristic. T. rhodesiense infection in mice is influenced by human serum, which has no therapeutic effect on the infection with T. gambiense in these animals. The organism has been cultivated on Novy and MacNeal's medium. There has been considerable controversy regarding the relationship of T. rhodesiense to T. brucei. While differences in the pathogenic effects of the two organisms have been observed, the right interpretation of the data constitutes a difficult question. Bruce and his co-workers, founding largely on extended biometric investigations, are of opinion that the T. rhodesiense is a strain of T. brucei which has adapted itself to man, and this view is now widely held. Other strains of trypanosomes have occasionally been recognised in human cases, and the relationship of these to the more fully determined forms has been the subject of much investigation.

Both normal serum and the serum of infected animals have been found to possess trypanocidal and protective properties, and in certain cases a degree of immunity has been established as the result of treating infections with trypanocidal compounds. It has not hitherto been possible, however, to produce such a degree of immunity as could be utilised either for prophylactic or therapeutic purposes. The serum of an infected animal may manifest a specific agglutination reaction towards the infecting trypanosome. A point of great importance, as bearing on the occurrence of relapses, is that brief contact *in vitro* of the anti-serum with the parasites renders the latter serum-resistant. This is shown when the treated organisms are inoculated into animals rendered immune to the original strain; the infection develops in these as it would in a normal susceptible animal (Ehrlich, Roehl, and Gulbransen). On account of this tendency of trypanosomes to alter in their behaviour to antisera serological methods cannot be relied upon for distinguishing different species of those organisms.

Methods of Examination.—On microscopic examination the organisms may be found in the cerebro-spinal fluid, the blood, or the juice of glands. In the case of the first, about 10 c.c. of the

fluid should be centrifuged for fifteen minutes and the deposit placed under a cover-glass for examination; it is better to make a little cell on a slide by painting a ring of ordinary embedding paraffin, to place the droplet of fluid in its centre, and to support the cover-glass on the paraffin; in this way injury to the delicate structure of the organism is avoided. In fresh cerebro-spinal fluid the trypanosomes can be seen to be actively motile; the number in which they occur varies very much, and the same is true to a greater degree of the blood, in which they are, however, usually very scanty. With regard to the examination of the blood, Bruce and Nabarro state that it is difficult by ordinary centrifuging to concentrate the organisms, as these are not readily precipitated. They accordingly recommend that the blood be mixed with citrate of sodium solution (equal parts of blood and of a 1 per cent. citrate solution) and centrifuged for ten minutes, that the plasma be removed and centrifuged again for the same time, and that this be repeated three times, the deposit on each occasion after the first centrifuging being carefully examined. Greig and Gray have insisted that the examination of the glands in a suspected case forms the most ready means of arriving at a diagnosis, and this opinion has found strong support from the work of Dutton and Todd. The method is to puncture the gland with a hypodermic needle, suck up a little of the juice, and blow it out on to a slide. In all cases where films of any kind are to be prepared the staining methods of Leishman or Giemsa are to be recommended. Often in cerebro-spinal fluid and gland juice the staining of the chromatin is difficult, but good preparations are obtained by the procedure recommended by Leishman for studying the parasite in sections (p. 676). The presence of scanty parasites may be shown by inoculating a rat or mouse with blood or cerebro-spinal fluid from a suspected case. The animal's blood should then be examined at intervals of several days for the next month or two. (This is readily done by snipping a small piece off the skin of the tip of the tail, and expressing a drop of blood.) It must be noted, however, that negative results have no significance, as an infection may fail to develop in the animal even when trypanosomes can be detected by microscopic examination in the inoculated blood.

Trypanosoma cruzi.—Apart from sleeping sickness, another important disease of man associated with trypanosomal infection occurs in Brazil, where Chagas observed the trypanosome in a monkey, the intermediate host being the reduviid bug, *Triatoma megista* (*Conorhinus*) (Fig. 189, 12–14). As these insects also feed on man, the possible relationship of the trypanosome to a human disease occurring in that region was considered. This disease affects children, and gives rise to pronounced anæmia, the occurrence of œdema, and enlargement of lymphatic glands, the spleen, and liver; it may cause death in a few weeks, or assume a chronic form lasting for years and characterised by disorders of internal secretion—myxœdema, bronzing of skin, and infantilism. The trypanosome is not found in large numbers in the

peripheral circulation in such cases, but when the blood is injected into guinea-pigs, or into callithrix monkeys, a definite disease occurs, leading to death. The special feature of interest is the multiplication of the parasite, which does not occur in the blood but in the tissue cells. Thus *post mortem* in man, the parasite is found chiefly in the cardiac and voluntary muscles and in the central nervous system, in which situations the tissue cells may contain enormous numbers of the organism in a leishmania form, which is the stage of multiplication (Fig. 189, 15); finally the trypanosome form is regained and the parasites become free through rupture of the cell. Similar appearances are met with in infected animals. A process of schizogony has also been described within endothelial cells in the lungs of infected guinea-pigs; it appears, however, that the latter structures were derived from the presence of another parasite in addition. A cycle of development takes place in the intestinal tube of the Conorhinus, and the infection is transmitted probably through contamination of wounds in the skin by the fæces of the bug. Cultures are obtainable on Novy and MacNeal's medium. The armadillo is probably the usual vertebrate host.

Trypanosomiasis in Animals

Nagana or Tsetse Fly Disease.—This disease deserves special mention since, although it affects animals chiefly, there is little doubt that a proportion of cases, if not all, are due to a trypanosome (T. rhodesiense) which is capable of infecting man Under natural conditions chiefly horses, cattle, and dogs are attacked; it is prevalent especially in certain regions of South Africa, though it probably may occur elsewhere. In the horse the chief symptoms are the following. The animal is observed to be out of condition, its coat stares, it has a watery discharge from the eyes and nose, and the temperature is elevated; swellings appear on the under surface of the abdomen and in the legs; it gradually becomes extremely emaciated and anæmic, and dies after an illness of from two or three weeks to two or three months. In other animals the symptoms are of the same order, though the duration of the disease varies much; thus in the dog the illness does not last more than one or two weeks, while in cattle it may continue for six months. It is doubtful whether a domestic animal attacked by the disease ever recovers. The popular idea regarding the etiology of the disease was that it was contracted by animals passing through certain rather restricted and sharply defined areas or belts characterised by heat and damp, sometimes lying beside rivers, and always infested by the tsetse fly (*Glossina morsitans*), to the bite of which the disease was attributed. In this connection it is important to note that though man is frequently bitten by the tsetse fly he only rarely becomes infected with these trypanosomes. Modern knowledge on nagana dates from the discovery made by Bruce in 1894 that the blood of affected animals swarmed with

trypanosomes. It was found that the parasite was present in the blood of every animal suffering from nagana and absent from the blood of healthy animals in the affected districts; further, the fever which marks the onset of the disease was shown to be accompanied by the appearance of trypanosomes in the blood; and finally, it was proved that the transference of a minute quantity of blood from an affected to a healthy animal originated the disease. As regards the part played by the tsetse fly in the condition, Bruce found that if flies taken from the fly belt, but which had not fed on an infected animal, were transported to a place where nagana did not occur, kept for a few days, and then allowed to bite susceptible animals, the latter did not contract the disease—this result showing that it was not, as had been supposed by some, a poison natural to the insect which was the pathogenic agent. But if such a fly was allowed to bite a dog suffering from the disease and then to bite a healthy dog, the latter contracted the malady and abundant trypanosomes were found in its blood. Again, threads dipped in the blood of an infected animal and allowed to dry caused the disease in healthy animals up to, but rarely beyond, twenty-four hours after being dried. If, however, the blood were kept moist, then it retained its infectiveness up to between four and seven days; up to forty-six hours living trypanosomes could be seen in the tube of the fly's proboscis. Further, Bruce showed that infection did not occur by any food or water partaken of by an animal while going through a fly belt, for he took horses through such a region without allowing them to eat or drink, and found that they still contracted the infection, if during their few hours' journey through the belt they had been bitten by the tsetse fly. Finally, he showed that if flies were taken from an infected area to a healthy one a few miles off and allowed to bite healthy animals, the latter contracted nagana.

By those experiments it was thus determined that nagana could be transmitted by the blood of the infected animal—that is, without the agency of the fly; that the latter had no inherent power to produce the disease; that it could, however, by successively biting infected and healthy animals, transmit the disease to the latter; and that specimens of the insect caught in infected areas harboured the parasite and were thus infective. The question remained as to how the flies might become infected in nature. It had been observed that in districts where the tsetse fly lived, the prevalence of the disease in imported animals was related to the presence in the locality of wild herbivora. Bruce now found that, if considerable amounts of the blood of the latter were taken to another locality and injected into dogs, these in a proportion of cases contracted nagana, and from this it was deduced that the wild animals harboured the parasites in small numbers in their blood and so acted as a reservoir of infection. Bruce's work as a whole pointed to the trypanosome as the cause of nagana, and this has since been finally established by producing the disease through the agency of flies bred under laboratory conditions and experimentally infected, as well as by artificial cultures of the organism. The trypanosome undergoes a cyclic development in the body of the insect similar to that of T. gambiense.

The trypanosome originally recovered by Bruce was investigated as regards its microscopic characters by Plimmer and Bradford,

who named it *T. brucei* (Fig. 190). It was found later to differ in its morphology from the trypanosome found subsequently by Bruce and others to be commonly associated with the disease nagana, the latter having the characters of *T. rhodesiense,* whereas the original T. brucei is monomorphic and resembles T. evansi (Stephens and Blacklock). It remains unsettled as to whether these differences indicate specific characters or whether the original T. brucei became altered as the result of prolonged sojourn in laboratory animals. Novy and MacNeal succeeded in cultivating

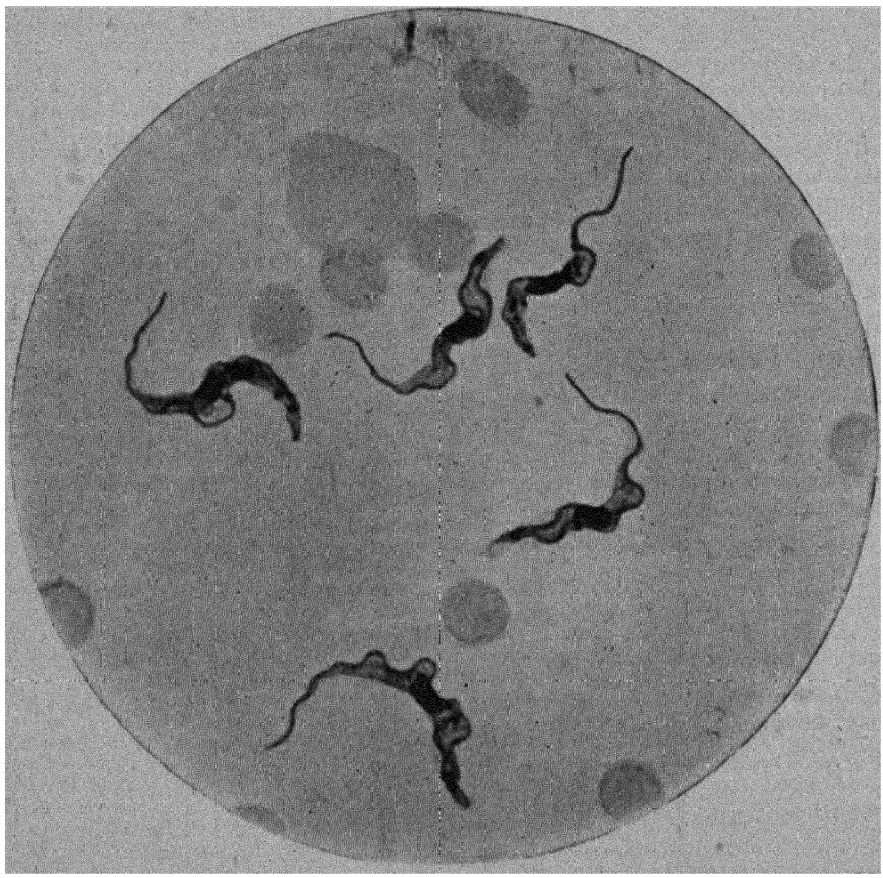


FIG. 190.—Trypanosoma brucei from blood of infected rat. Note in two of the organisms commencing division of micronucleus and undulating membrane. ×1000.

this trypanosome, though it was very difficult to obtain a first growth from the blood on their blood agar medium ; once started, however, it was maintained through many subcultures, the optimum temperature of growth being 25° C., and it was from these subcultures that the infection was obtained which definitely proved the organism to be the cause of the disease.

Nearly all laboratory animals are susceptible to infection, and the duration of the illness corresponds to what has been observed in the natural infection of these animals. But repeated passage through a particular species tends to increase the virulence of the trypanosomes for that species. Thus T. brucei when accommodated

to mice leads to death of these animals with septicæmia in three to four days after inoculation.

Serious disease in cattle, horses, sheep, and other domestic animals in Africa is caused also by T. congolense and T. vivax. *T. congolense* is a small monomorphic trypanosome devoid of flagellum which measures 9–18 μ by under 3 μ. It is transmitted by Gl. morsitans and other tsetse flies. This trypanosome does not invade the salivary gland of the fly, but passes from the stomach to the labrum. *T. vivax* is distinguished by its active movements of progression; it measures 18–26 μ in length, has a definite flagellum, and the macronucleus lies toward the anterior end of the body. In the tsetse fly (Gl. morsitans and other species) multiplication of the trypanosomes occurs only in the proboscis.

It is beyond the scope of this work to deal at length with the other trypanosome infections of animals, but it may be said that many species have been described in various mammals, birds, and fishes, and that these are spread either by insects or by leeches. One of the most interesting is Dourine, a condition resembling in many ways nagana, caused by *T. equiperdum*. It, however, presents this peculiarity, that infection does not take place by an intermediate host, but apparently directly through coitus, as it occurs only in stallions and in mares covered by these. Surra is also due to a trypanosome (*T. evansi*); although first observed in horses in India, the infection is widely spread in Asia, Africa, and the Philippines, and severely affects domestic animals and camels. The infection appears to be spread directly by biting flies (various species of Stomoxys and Tabanus). Mal de Caderas, a disease of horses in South America, is also due to a trypanosome (*T. equinum*). Biting flies are believed to transmit this infection also. The parasites of dourine and surra resemble the slender forms of T. gambiense. T. equinum is similar in size, but in it there is only a minute blepharoplast and the parabasal body is absent. The trypanosome found in cattle in Africa and other parts of the world (*T. theileri*) is of doubtful pathogenicity. *T. melophagium*, a non-pathogenic trypanosome of wide occurrence in sheep, is transmitted by the sheep-ked, in the hind-gut of which the infective forms of the parasite develop.

Trypanosoma lewisi.—This trypanosome is very common in the blood of rats all over the world, though the percentage of animals affected varies in different localities. The organism has no importance from the standpoint of human pathology, but it is of great interest that the infection runs a very definite course in the rat, although it is very rarely fatal. A fatal issue may, however, occur in young individuals, especially when these are infected with strains of the organism imported from other localities. The trypanosome, as it appears in the blood in the later stage of the infection, is actively motile, is of ordinary length but is somewhat narrow, the posterior end is pointed, the macronucleus lies in front of the centre of the body, and the protoplasm does not contain any granules. It multiplies by fission, of which Laveran describes two varieties. In one, the organism splits longitudinally and gives rise to smaller individuals than the parent. In the other, the trypanosome loses its ordinary shape and becomes more oval: nuclear division, which is often multiple, then takes place, and on subsequent division of the protoplasm a number of small flagellate

organisms result; these last may attain the full form and size before dividing again, or they may divide when still small. When a rat is infected by injection into the peritoneum, active multiplication goes on in the cavity for a few days and then comes to an end. Very soon after infection the organisms begin to appear in the blood, and there rapid multiplication occurs, the extent of which is sometimes so great that the trypanosomes may seem to equal the red blood corpuscles in number. The animal usually shows no symptoms of illness. The infection goes on for about two months, and then the organisms gradually disappear from the blood In the great majority of cases the rat is now immune against fresh infection. If trypanosomes be introduced into its peritoneum they are, according to Laveran, taken up by mononuclear phagocytes and destroyed. The serum of a rat which has been infected shows agglutinating action on the trypanosomes, causing them to agglomerate in rosettes in which the flagella are directed outwards; the serum of immune rats has also a certain degree of protective action if injected along with the organisms into a susceptible animal As has already been noted, this trypanosome has been cultivated in artificial media, on which it multiplies freely, large numbers of small forms being often produced (the optimum temperature is about 20° C.). These when injected into rats give rise to the usual infection. Novy and MacNeal succeeded in carrying a growth through many subcultures. The trypanosome is very resistant to cooling, and has been exposed for fifteen minutes to the temperature of liquid air (- 191° C.) without being killed. Rabinowitsch and Kempner have shown that the rat flea, *Ceratophyllus fasciatus*, transmits the parasite, infection occurring through the fleas or their fæces being swallowed (Nöller, Minchin and Thomson, and others). In the flea a phase of multiplication of the trypanosomes occurs within the epithelial cells of the stomach; the parasites then migrate to the hind-gut and rectum, assume the crithidial form and undergo fission, and finally take on the trypanosome form again. The flea becomes infective about a week after biting, and remains infective for the rest of its life. Infection may also take place through other species of fleas and through the rat louse (*Hæmatopinus spinulosus*).

Leishmaniasis

Under this term there are grouped three human diseases caused by protozoal parasites whose exact zoological place is not yet definitely settled. These organisms are the *Leishmania donovani*, associated with the human disease kala-azar; *Leishmania infantum*, derived from a similar disease occurring in children; and *Leishmania tropica*, which has been found in a skin ulceration of widespread geographical distribution. Microscopically the organisms are practically identical, but it is probable that they belong to two, or possibly three, distinct species. A similar parasite is associated with a disease in dogs, the symptoms of which in many respects resemble those met with

in the human subject. The geographical distribution of canine leishmaniasis partly corresponds with that of the human varieties, but the association of leishmania infections in the dog and the human subject is by no means invariable.

Leishmania donovani.—Leishman noticed in several soldiers invalided from India for remittent fever and cachexia that very careful examination of the blood failed to reveal the presence of the malarial parasite. Most of these patients had been quartered during their service at Dum-Dum, an unhealthy cantonment near Calcutta, and from this fact the existence of a disease hitherto undescribed was suspected. In 1900 Leishman found in the spleen of such a case peculiar bodies, which resembled degenerating forms of T. brucei, and he suggested that they might be trypanosomes. Leishman's observations were confirmed in India by Donovan, and the bodies associated with the disease are now usually called the " Leishman " or the " Leishman-Donovan " bodies. They were found by Bentley, and later by Rogers, in the disease known in Assam as kala-azar, the pathology of which had long been obscure, since, while it resembled malaria in many ways, no malarial parasites could be demonstrated. This disease has gone under various synonyms, *e.g.* cachectic fever, Dum-Dum fever, non-malarial remittent fever, but is now recognised as a single specific entity.

Kala-azar (or " black disease,"—so called from the hue assumed by chocolate-coloured patients suffering from it) has been known since 1869 as a serious epidemic disease in Assam, where it has spread from village to village up the Brahmaputra valley. The disease is now known to occur in various subtropical centres—cases where the Leishman bodies have been found having been met with in many parts of India, China, Turkestan, the Malay Archipelago, North Africa, the Soudan, Syria, and Arabia. The disease is characterised by fever of a very irregular type, by progressive cachexia, and by anæmia associated with enlargement of the spleen and liver, and often with ulcers of the skin and with transitory dropsical swellings. Some cases at the commencement may resemble enteric fever. Rogers has pointed out that there occurs a leucopenia which differs from that of malaria in that it is almost always more marked,—the leucocytes usually numbering less than 2000,—and further, in that the white cells are always reduced in greater ratio than the red corpuscles, which condition, again, does not occur in malaria. The disease is chronic, often going on for several years, and in, at any rate, 80 per cent. of the cases has a fatal issue. *Post mortem,* there is little to note beyond the

enlargement of the liver and spleen, but in the intestine, especially in the colon, there are often large or small ulcers, and there is evidence of proliferation in the bone marrow, the red marrow encroaching on the yellow.

In a film made from the spleen and stained by Leishman's stain, the characteristic bodies can be readily demonstrated (Fig. 191). They are round, oval, or, as Christophers has pointed out, cockle-shell-shaped, and usually 2·5 to 3·5 μ in

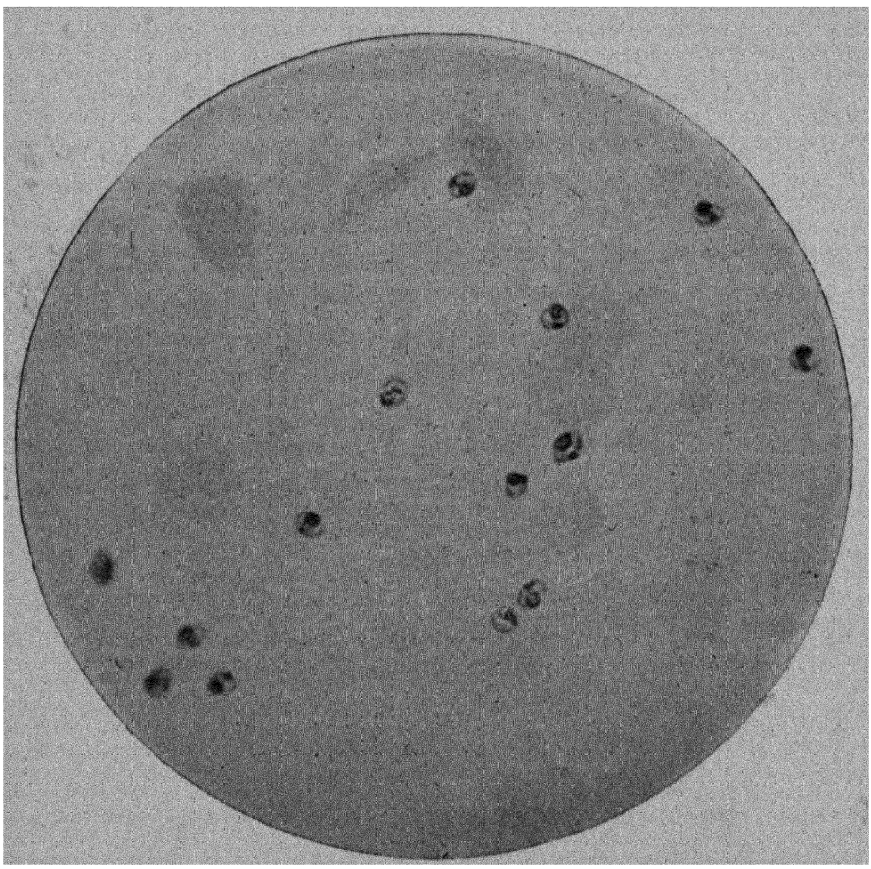


FIG. 191.—Leishman-Donovan bodies from spleen smear. ×1000.

diameter, though smaller forms occur. The protoplasm stains pink, or sometimes slightly bluish, and contains two bodies taking on the bright red colour of nuclear matter when stained by the Romanowsky combination. The larger (trophonucleus), which stains less intensely than the smaller, is round, oval, heart-shaped, or bilobed, and lies rather towards the periphery of the body—in the region of the "hinge" in the cockle-shaped individuals. The other chromatin body (kinetonucleus or kinetoplast) is usually rod-shaped, and is set perpendicularly or at a tangent to the larger mass, with which only exceptionally it

appears to be connected. According to Christophers, Shortt, and Barraud, the smaller chromatin body is differentiated into the parabasal body, and, running at right angles to the long axis of the latter, the rhizoplast (axoneme), which is a straight or slightly curved linear structure measuring about half the diameter of the parasite in length. The aspect of the parasite on which the parabasal body is situated is taken to be dorsal, and from it, at a point corresponding to the blepharoplast, the rhizoplast extends anteriorly. Usually the protoplasm contains one or two vacuoles. Though in spleen smears many free bodies are seen, the study of sections shows that ordinarily their position is intracellular,—the cells containing them being of a large mononuclear type (Fig. 192). The view held is that on their entering the circulation they are taken up by the mononuclear leucocytes and by such cells as the endothelial lining of the splenic sinuses or those lining capillaries or lymphatics, that in these cells multiplication takes place,—it may be to such an extent as to rupture the cell,—and that if thus the bodies become free they are taken up by other cells and the process is repeated. The clusters of bodies sometimes seen in smears are probably held together by the remains of ruptured phagocytes. In capillaries the endothelial cells after phagocyting the bodies probably become detached from the capillary wall, as they are often observed free in the lumen of the vessel—this being well seen in the hepatic capillaries. The parasites are found in greatest abundance in the spleen, liver, and bone marrow, and also in mesenteric glands, especially in those draining one of the intestinal ulcers; less frequently they occur in the skin ulcers, and in other parts of the body, *e.g.* the villi of the intestine may be crowded with them (Perry). Donovan described them as occurring in the peripheral blood, especially within the leuco-

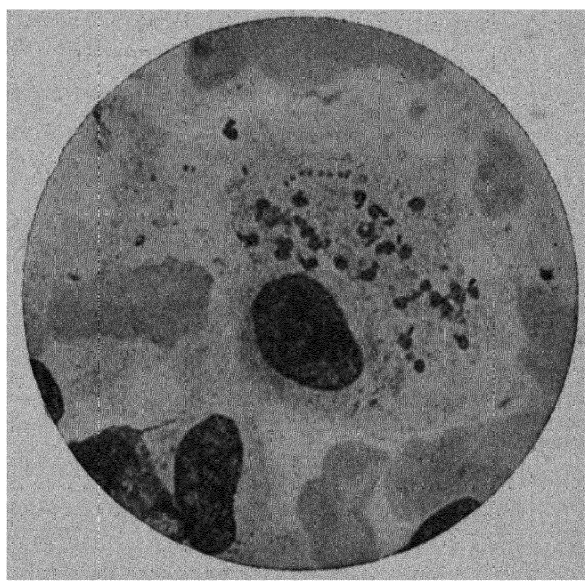


FIG. 192.—Leishman-Donovan bodies within endothelial cell in spleen. See also Plate VI., Fig. 24. ×1000.

cytes, and this observation has been generally confirmed, though sometimes prolonged search is necessary. Patton has found that the numbers in the blood vary from time to time, and special incursions may be associated with exacerbations of dysenteric symptoms which he holds indicate a spread of the intestinal ulceration. Shortt and his co-workers have found that the organisms can be frequently recovered in cultures made from the urinary sediment.

In the body the parasite multiplies by simple fission, both nuclei dividing amitotically, and two new individuals being formed; but sometimes a multiple division takes place, each nucleus dividing several times within the protoplasm and a corresponding number of new parasites resulting.

In view of Leishman's original opinion, an extremely important discovery was made by Rogers, to the effect that in cultures a flagellate organism developed from the Leishman-Donovan body. Cultivation was effected by taking spleen juice containing the parasite, placing it in 10 per cent. sodium citrate solution, and keeping it at 17° to 24° C. Under such conditions there occurs an enlargement of the organism, but especially of the larger nucleus. This is followed by the appearance of a pink-staining vacuole in the neighbourhood of the smaller nucleus. Along with these changes, in from twenty-four to forty-eight hours the parasite becomes elongated and the smaller nucleus and its vacuole move to one end; from the vacuole there then appears to develop a red-staining flagellum, which when fully formed seems to take its origin from the neighbourhood of the small nucleus. The body of the parasite is now from 20 to 22 μ long and 3 to 4 μ broad, with the flagellum about 22 μ long. The whole development occupies about ninety-six hours. The formation of an undulating membrane was not observed, and, although the flagellated organism moved flagellum first, like a trypanosome, it is evident that here the relationship of the micronucleus is different, as this structure lies anterior to the macronucleus. The serum of many animals, *e.g.* man, guinea-pig, has an inhibitory effect on the parasite, but success in the cultivation has attended the use of Novy and MacNeal's medium made up with rabbit's blood (that of the sheep or dog may also be used). The phases of the parasite in such cultures have been investigated by Christophers, Shortt, and Barraud; they concluded that the masses of mature flagellate forms which develop from the Leishman-Donovan bodies leave the group where they were produced and become free-swimming. These separate and later come to rest; then as a result of

repeated divisions each gives rise to a number of short multiplicative forms, some of which again are provided with vibratile flagella and, becoming free-swimming, repeat the cycle. In cultures which have been kept some time (at least seven days), small rounded flagellate forms appear which measure about 2 μ; these are considered by Row to be of the nature of cysts. In old cultures Leishman described the occurrence of unequal division of the parasite, which resulted in the splitting off of a hair-like undulating form containing a chromatin granule; the final development of these spirillary forms has not been traced.

As regards the classification of this organism, which now usually goes by the name Leishmania donovani, given to it by Ross, although its flagellated form is that of a *leptomonas* [1] (Fig. 193), and so differs from the typical trypanosome form, it bears considerable resemblance to the members of this group. But as Leishman has pointed out, the cultures may not represent the full development of the organism in the trypanosome direction. Minchin's suggestion has been accepted, however, that in the present incomplete state of knowledge it is well to place it and its congeners in a provisional genus, Leishmania, of the flagellata.



FIG. 193.—*Leishmania donovani.* Leptomonas form from culture. Leishman's stain. ×1000.

Experimental Infections.—Though results obtained in different parts of the world vary somewhat, certain animals (*e.g.* monkeys, young dogs, and mice) have, in a varying proportion of cases, been infected with the parasite as it occurs in human lesions and also in cultures. It is of importance that young cultures may be incapable of transmitting the infection, whereas those which are older (seven to thirty-three days) are infective, and this may be related to the development in the culture of special forms of the parasite (Row's torpedo forms). The intraperitoneal route is the best, and both when the animals have died and have been killed Leishmaniæ have been found in such

[1] In the leptomonas the blepharoplast is situated at the anterior end, and there is no undulating membrane.

situations as the spleen, the liver, the bone marrow. Recovery from the infection is followed by immunity to re-inoculation. Feeding experiments have usually been unsuccessful, but one or two positive results are recorded. In India the examination of dogs which have been in contact with kala-azar cases has not yielded evidence that natural infection occurs in these animals.

The question arises, given that the Leishmania donovani is the cause of kala-azar, how is infection spread ? In the absence of precise knowledge, a number of theories have been held. Ingestion of the parasite has been stated to produce infection under experimental conditions, but the recent extensive work of the Indian Kala-azar Commission with mice lent no support to this view ; further, the existence of a resistant form of the parasite which would survive in the alimentary tract has not been demonstrated. Supposing that infection could occur by this route, it is necessary to consider its source ; attempts to discover the Leishmania bodies in the fæces of cases have not been successful, and no other probable source of the parasites has been substantiated. Again, the possible relationship of the organism to the trypanosomes suggested that an insect might act as the intermediary host, and Rogers adduced evidence that the bed-bug is the extra-human host. Patton fed the bug, *Cimex rotundatus*, on patients whose peripheral blood contained the Leishmania, and observed the flagellate stage. It appears, however, from the work of F. P. Mackie and others, that bugs obtained from the bedding of kala-azar patients, or fed upon such cases, very rarely contain flagellates, and material from such bugs when injected into monkeys does not produce the infection. Numerous other insects have been suspected, but without definite evidence being obtained that they play any part in the transmission of the disease. A frequent difficulty in such work has been the presence of a variety of flagellates which normally inhabit the intestines of insects. Recently the Kala-azar Commission has brought forward strong evidence that a sandfly, *Phlebotomus argentipes*, is the insect host. When flies of this species were fed upon cases of kala-azar, flagellates appeared in the intestine of 25 per cent. In view of the scantiness of Leishmania in the peripheral blood of patients, such multiplication in this fly indicates that it affords a specially favourable medium for the parasite. The appearances met with in the intestinal contents of the infected flies were the same as those found in artificial cultures of Leishmania ; further, a large number of flies fed upon healthy individuals failed to show flagellates. It is of importance also that in several

instances the Commission carried out control experiments in which the same cases as caused infection of *P. argentipes* were fed upon by bugs, but with entirely negative results. Infected sandflies showed a marked infection of the gut five days after feeding upon a case, and in a considerable proportion of flies which survived to the eighth day the pharynx and buccal cavity contained flagellates, but they did not become established to any extent in the rectum. Thus it would appear that the parasites are conveyed to the human host by the fly in the act of biting. The conclusive experiment of transmission of the infection by the fly has not yet been carried out. It is, of course, not excluded that other blood-sucking insects may also carry the infection in different parts of the world.

Methods of Examination.—The Leishman-Donovan bodies can be readily seen in films or sections of the infected organs. These should be stained by the Romanowsky stains. Fluid taken from the enlarged spleen with a perfectly dry needle during life may be examined, but it is probable that in this disease puncture of the spleen may not be a very safe operation, as death from hæmorrhage from this organ is not uncommon ; liver-puncture is less dangerous, but the parasites are not so readily found in this way ; the juice of enlarged lymphatic glands may also be examined. The demonstration of the parasite in the circulating blood should always be attempted by means of ordinary as well as thick films. Another method consists in inoculating a number of tubes of Novy and MacNeal's (N.N.N.) medium each with several drops of blood from the patient ; cultures should be examined after a few days, and again after two or three weeks. The absence of malarial parasites from the blood and the leucopenia which has been alluded to are also important features.

Leishmania infantum.—Nicolle, working in Tunis, observed a disease clinically identical with kala-azar, affecting children between two and five years of age. He found in the spleen, liver, and bone marrow in such cases an organism microscopically indistinguishable from the Leishmania donovani. The disease is very widespread, and occurs along the whole of the south and east littorals of the Mediterranean, in Portugal, Greece, Sicily, and in Italy as far north as Rome, in the Soudan and Abyssinia. The organism can be cultivated on a modified form of Novy and MacNeal's medium (p. 680).

The cultures present characters similar to those observed by Rogers and Leishman in the other Leishmaniæ. It has been found that the organism can be successfully inoculated in the

dog, monkey, mouse, rat, guinea-pig, and rabbit by intrahepatic and intraperitoneal injection of spleen pulp from fatal human cases; and Novy and MacNeal have produced the disease by inoculation with massive doses of cultures. The fact that animals had not, up to the time of his observations, been infected with the Leishmania donovani, and the further fact that the disease, as it occurs in the regions named, is apparently confined to young children, led Nicolle to look upon the organism as a separate species to which he gave the name *Leishmania infantum.* He considered the infection of the dog to be significant, as this animal might be the channel through which children become infected, for in most regions where the disease prevails there occurs a disease of dogs, which may be either of an acute or chronic character, and which is apparently due to an identical organism. Laveran found that a macacus monkey which had recovered from an infection with Leishmania derived from Tunis was also immune to inoculation with parasites obtained from a case of Indian kala-azar; thus it would appear likely that the two viruses are identical, although they show differences in virulence and resistance. The view has been advanced that in the case of *L. infantum* infection takes place by the agency of fleas, but experimental evidence does not support this.

Leishmania tropica.—In various tropical and sub-tropical regions (India, Central Asia and the East, Northern Africa, Southern Russia, Turkey, South America, West Indies) there is widely prevalent a variety of very intractable chronic ulceration which goes by various names in different parts of the world—Delhi sore, tropical ulcer, Aleppo boil, etc. These sores may spread from one part of the body to another by auto-inoculation, also they may be communicated to other individuals by contact. The work of J. H. Wright first showed that a protozoal parasite is concerned in the etiology of the condition. In the discharge from the ulcer and in sections of a portion of tissue excised from a case coming from Armenia, Wright observed great numbers of bodies which are indistinguishable morphologically from the Leishman-Donovan body. It was found that the bodies were usually intracellular in position in the lesion, as many as twenty being in one cell, and that the type of cell containing them was, as in kala-azar, that derivable from endothelial tissues.

Wright's observations have been fully confirmed by workers in various parts of the world, and it is now recognised that in these tropical ulcers we have another example of the activity of a Leishmania. The duration of the sore is about a year. It is stated that after recovery the individual possesses immunity.

Sometimes the parasite is destroyed in a foul ulcer, but can still be obtained by taking some of the juice from the marginal indurated tissues by capillary glass tubes. Patton reports having found the organism in the blood taken from parts adjacent to the ulcer. The organism may be grown in Novy and MacNeal's (N.N.N.) medium ; the appearances in culture are considered by some to differ from those met with in the case of the Leishmaniæ already described. Thus *L. tropica* tends to develop forms with two flagella, which are stated not to occur with *L. donovani* or *L. infantum.* Row has obtained cultures in citrated blood. Nicolle and Manceaux reproduced the condition in man, the monkey, and the dog, both by virus obtained from the natural infection and from cultures on Novy and MacNeal's medium. The lesions were identical with those naturally occurring, the incubation period being often many months. In the male mouse intraperitoneal injection is followed either by a granuloma in the testicle, or by a generalised infection in which lesions often characterised by widespread destruction of tissues occur in the skin or around the joints ; all these lesions contain numerous parasites. Various views have been held as to how infection takes place ; Patton believed the bed-bug to be the intermediate host, perhaps exclusively during its nymph stage. Recently it has been proved that the disease is transmitted by phlebotomus flies, as was shown by Adler and Thornton in Palestine. They inoculated into the skin in a human subject the contents of the alimentary tract of P. papatasii which contained throughout its length numerous leptomonas forms ; a month later a small papule had developed at the site of inoculation which contained Leishmaniæ. Thompson and Balfour have described in the Soudan a condition in which subcutaneous nodules without ulceration occurred in man, and these contained Leishmania bodies. In the South American form of the disease, after the skin ulcers have appeared, typical lesions occur not uncommonly at a later date in the nasal and buccal mucous membranes ; such cases are liable to develop a severe cachexia. It has been suggested that this condition is due to a specific parasite, *L. braziliensis.*

The close similarities in the morphology and effects of the three Leishmaniæ naturally raise the question whether we are not dealing with variants of one organism whose differences depend on differences in the virulence of different types or on the susceptibility of different hosts. The following are some of the facts bearing on this question. The Indian and Mediterranean diseases are apparently clinically identical, and while on the

one hand in certain parts of India kala-azar is chiefly found in children below the age of fifteen, on the other hand cases occur in young adults in regions where the infantile variety prevails. The importance of such factors as racial susceptibility is indicated by the fact that in Tunis it is chiefly the children of Italian parentage who suffer. Kala-azar and Oriental sore are linked by the occurrence in the former from time to time of skin ulcers, although in these, unlike the case of Oriental sore, the parasites are difficult to find ; on the other hand, it is of importance that cases of Oriental sore do not develop into kala-azar. Again, Nicolle found that dogs infected with Leishmania tropica appeared to be less susceptible to Leishmania infantum than usual. The incidence of canine Leishmaniasis in communities where a human infection prevails varies in different regions, *e.g.*, even in the Mediterranean littoral, though it is usually common where Leishmania infantum is found. Laveran, as has been stated, held the view that the kala-azar of India and the human disease occurring in the Mediterranean are identical with the disease of dogs. Recently the problem of the relationships of the Leishmaniæ has been investigated by serological methods (Noguchi, Kligler). It has been found that antisera obtained from rabbits by injecting intravenously cultures of the organisms possess both agglutinating and lytic action. The results obtained by the use of such sera indicate that L. infantum, L. tropica, and L. braziliensis are specific organisms, but more extended investigations are required.

Piroplasmosis

Up to the present no human disease has been proved to be associated with the presence of piroplasmata. But several important diseases of the lower animals are caused by protozoal parasites of this group, and a short account of the organisms may be given.

The piroplasmata are pear-shaped unicellular organisms which vary in size according to the species from 3–4 μ in length down to less than 1 μ. The peripheral part is denser than the central, which often appears as if vacuolated, and at the broad end there is a well-staining chromatin mass. Sometimes irregular and ring-, rod-, or oval-shaped individuals occur. The organisms are found within the red blood corpuscles of the infected animal and also free in the blood. In the former situation there is sometimes only one within a cell, but the numbers vary under different circumstances and in different species. No pigment is formed. Multiplication takes place by fission, and the new individuals, two or four in number, remaining for longer or shorter times in apposition, account for some of the appearances seen in cells. Especially in the forms free in the blood, pseudopodial prolongations of the protoplasm, usually from the pointed end, are developed, and it may be by means of such pseudopodia that entrance to the red

cells is obtained. Infection is usually carried from infected animals by means of ticks, as Smith and Kilborne showed. In one case Koch has described the development in the piroplasm, in the stomach of the tick, of spiked protoplasmic processes sprouting out from the broad end of the organism, and the occurrence of conjugation of two such individuals by their narrow ends to form a zygote. Observations by Christophers indicate that a globular body now appears, probably corresponding to the oöcyst stage of other similar protozoa, and the further development consists in a division into sporoblasts which may infect the whole tissues of the tick, especially the salivary apparatus. The eggs may also be infected and the young ticks developed from these may thus be capable of carrying the disease to fresh hosts (Smith and Kilborne). Frequently when an animal has passed through an attack of a piroplasmosis it is immune to the disease, and with regard to this immunity in certain cases very interesting facts have been observed. For instance, the condition may not be associated with the disappearance of the parasite from the blood of the immune animal, and the latter may thus be a source of danger to other non-immune animals with which ticks harboured by it may come in contact.

The following are some of the chief piroplasmata causing disease in animals: *Piroplasma bigeminum* (*Babesia bigemina*). This was first accurately described by Theobald Smith, and is the cause of Texas or red-water fever, a febrile condition associated with hæmoglobinuria, which occurs in the Southern States of America, South America, South and Central Africa, Algeria, various parts of Northern Europe, and in Australia. The organism gets its name of bigeminum from the fact that it is often present in the red cells in pairs, which may be attached to one another by a fine thread of protoplasm; this probably results from the complete separation of two individuals being delayed after division has occurred. It is the largest of the piroplasms found in cattle. Infection is spread by the tick *Boöphilus bovis* (*Margaropus annulatus*), and some of the characteristics of the disease epidemiologically are explained by the fact that this insect goes through all its moultings on the same individual host; but other ticks have also been shown to spread the infection. *Babesia bovis* is also a cause of red water in cattle; it is much smaller than *B. bigemina*. The tick *Ixodes ricinus* transmits the infection. *Piroplasma equi* gives rise to biliary fever in horses in South Africa. As was shown by Theiler, it is carried by the tick *Rhipicephalus evertsii*. Mules and donkeys can also be infected. Young horses are less severely affected than older ones; after clinical recovery the blood of such "salted" animals can be used for the protective inoculation of young horses. *Piroplasma canis* causes a piroplasmosis occurring in dogs. The organism has been cultivated by Thomson and Fantham and others by the method of Bass and Johns (p. 658).

Piroplasma parvum (*Theileria parva*).—This organism was discovered by Theiler in the blood of cattle suffering from African East Coast fever, a serious disease characterised by fever and enlargement of the lymphatic glands, but without the occurrence of hæmoglobinuria. The organism is small, appearing as ovoid or rod-shaped forms which measure 1–3 μ in length and 0·5–0·7 μ in breadth. *Rhipicephalus appendiculatus* and certain other ticks convey the disease. It is of interest that in the case of this organism, as contrasted with the other

piroplasms described above, division occurs not in the red corpuscles but in endothelial cells of the lymphatic glands, spleen, and other organs. The schizonts appear as masses of protoplasm 3–10 μ in diameter, containing a large number of minute chromatin dots The intracorpuscular forms of the parasite appear to be adapted for development in the tick, since inoculation of blood from an infected animal into other cattle does not produce the disease. With regard to the pathology of infection by piroplasmata we know nothing The diseases are often extremely fatal, carrying off nearly every individual attacked, the nature of the changes originated being unknown.

Oroya Fever

This disease, which occurs in Peru, is characterised by fever of intermittent type along with severe rapidly progressive anæmia, and is fatal in a large proportion of cases Although the causal agent now appears to be bacterial in nature, the condition may be considered here, since, as Barton found, the infective agents invade the red corpuscles. They are seen as minute coccal forms or as straight or slightly curved rods, measuring from 0·3 to 2 5 μ, which are motile in the fresh state ; when stained with the Romanowsky stains they have a reddish violet colour, the extremities being more intensely stained and often appearing thickened A large proportion of the red cells may be affected Strong and his co-workers of the American Commission gave the name of *Bartonella bacilliformis* to these bodies, which were considered to be probably protozoal and similar to *Theileria parva*. *Post mortem*, large collections of granules have been found in the vascular endothelial cells of the internal organs and especially of the lymph glands, which have been supposed to represent a stage in the multiplication of the parasite. Recently, Noguchi and Battistini have obtained cultures of the organism on solid or semi-solid media after inoculation with citrated blood from a case of the disease The organism possessed considerable resistance as it survived in the blood for 43 days. Cultures grew in leptospira medium (p. 587) and on sloped blood agar with a reaction corresponding to P_H 7·8, but not on ordinary agar or in broth. The most suitable temperature for growth was found to be 25–28° C. The organism as seen in cultures resembles the forms met with in the blood but is more pleomorphous and tends to form large clumps. It is Gram-negative and an obligate aerobe. In certain conditions it is motile, and flagella can be demonstrated by appropriate staining Intravenous inoculation of pure cultures into young *Macacus rhesus* monkeys produced intermittent fever of long duration, the characteristic organisms appearing in scanty numbers in the red cells ; but anæmia did not occur in these animals. Passages were effected from animal to animal, and the organism was recovered from the blood, lymph glands, and spleen. Filtered cultures were inactive. After intracutaneous inoculation into the eyebrows in young rhesus monkeys, vascular nodules developed which showed large numbers of the organisms situated within endothelial cells. The relationship of Oroya fever to *Verruga peruviana*, a disease characterised by a nodular skin eruption, has been much discussed; but according to the American Commission's results the two diseases would appear to be distinct.

CHAPTER XXIX

PATHOGENIC FUNGI

In pathological bacteriology, besides the bacteria themselves, higher organisms belonging to the group of fungi not infrequently claim attention. On the one hand, cultures may be contaminated with the spores of the omnipresent terrestrial forms growing in all decaying material, and, on the other hand, fungi of the same type are known to be the causal agents in certain diseases. Before considering the latter, with which we are more intimately concerned, we shall first give a short account of the group of fungi as a whole and of some of the common saprophytic forms.

The overwhelming majority of fungi consist of tubular branched filaments, termed *hyphæ*, each of which has a thin continuous wall within which are the protoplasmic and other contents. The whole body of the fungus thus composed of hyphæ is termed the *mycelium*. This may be loose and web-like in texture, as in the case of common moulds, or may assume the form of a compact skin or mass which is produced by the copious branching and close interweaving of the hyphæ, as in ordinary toadstools.

In the *Phycomycetes*, a lowly organised group of fungi, the hyphæ are typically continuous tubes devoid of any cross septa, excepting where reproductive organs or cells occur; whereas in the more highly organised fungi, *Mycomycetes*, the hyphæ are segmented by transverse walls.

Inasmuch as fungi have descended from algæ, which are mainly aquatic, those fungi that are most alga-like betray in their life-history signs of the aquatic mode of existence. Thus in a number of Phycomycetes the ends of certain hyphæ become shut off by a transverse wall. The terminal chamber becomes swollen and its abundant protoplasm divides into a number of cells, which, by rupture of the outer wall, escape as naked ciliated *swarm-spores*. Each of these swims about in water (raindrops and so forth), eventually clothes itself with a thin cell-wall, and, emitting a hypha which grows and branches, develops into a new plant. The terminal organ within which these *asexual spores* arise is termed a *sporangium*. In other types of Phycomycetes, for instance *Mucor Mucedo* (Fig. 194), the spores arising in the same manner inside a sporangium acquire a cell-wall before rupture of the sporangium wall: in this case the walled spores are not swarm-spores, but are adapted for dispersal through the air.

Some of the Phycomycetes can produce spores asexually in an

entirely different manner, namely, externally by abstriction from the end of a hypha. Such asexual spores externally cut off are termed *conidia*, and the special hypha bearing the conidia, if different in form from the vegetative hyphæ, is termed a *conidiophore*. Each conidium can emit one or more hyphæ and thus give rise to a new plant.

Other forms of asexual spores occurring in these simple fungi include *oidia*, in which a hypha undergoes cross septation into a number of short segments, each of which acts as an asexual spore. A hypha in this oidial condition has a resemblance to a greatly magnified row of bacteria ; indeed according to one theory bacteria represent merely oidial conditions of very degenerate fungi.

Finally, as opposed to the thin-walled asexual spores so far mentioned, thick-walled asexual spores (often termed *chlamydospores*) occur in some of these simple fungi, and are endowed with greater powers of resistance to hostile external conditions and act as resting-spores.

Phycomycetes also reproduce sexually. In the simplest case, as represented by *Mucor Mucedo*, the ends of two hyphæ come into contact and the terminal parts of the hyphæ are segmented off by a transverse wall. The wall at the region of contact of the two hyphæ is dissolved, and the protoplasmic contents of the two terminal compartments fuse and produce around the resultant mass a thick wall. This thick-walled structure is capable of growing out to produce a new plant. As it is produced by the fusion of two structurally similar sexual cells it is termed a *zygospore*. Those Phycomycetes that have no marked structural distinction between male and female cells or organs, and whose sexually produced cells are therefore zygospores, are grouped together to form the class *Zygomycetes*. In some species of Zygomycetes, despite the similarity of the sexual organs, certain individuals (e.g. *Mucor Mucedo*) are physiologically unisexual, being either + (female) or — (male), and a zygospore results only when the hyphæ of the two such individuals fuse : in other species the individual is bisexual and can produce zygospores by the fusion of its + and — sexual organs.

In other Phycomycetes there is a very clear distinction between, on the one hand, the large female organ, which encloses one or more female cells, the ova or *oospheres*, and, on the other hand, the usually smaller but differently shaped male organ, which contains the equivalent of a number of male cells. The union of some of the protoplasm, including nuclear material, of the male organ with an oosphere results in the production of a fertilised egg-cell or *oospore*. Those Phycomycetes having this mode of sexual reproduction are grouped together to form the class *Oomycetes*.

Sexually produced cells, zygospore and oospore, germinate vegetatively to produce a new mycelium or in a fructificative manner to produce a sporangium. Now the number of spores inside a sporangium of a Phycomycete is often not only considerable but also variable in the same species. Thus if a plant of *Mucor Mucedo* be starved, the number of spores produced in each sporangium is reduced. Similarly in the Phycomycetes the number of conidia produced on a conidiophore is considerable and variable. Sporangia and conidiophores, then, are *indefinite* in type in these simple fungi.

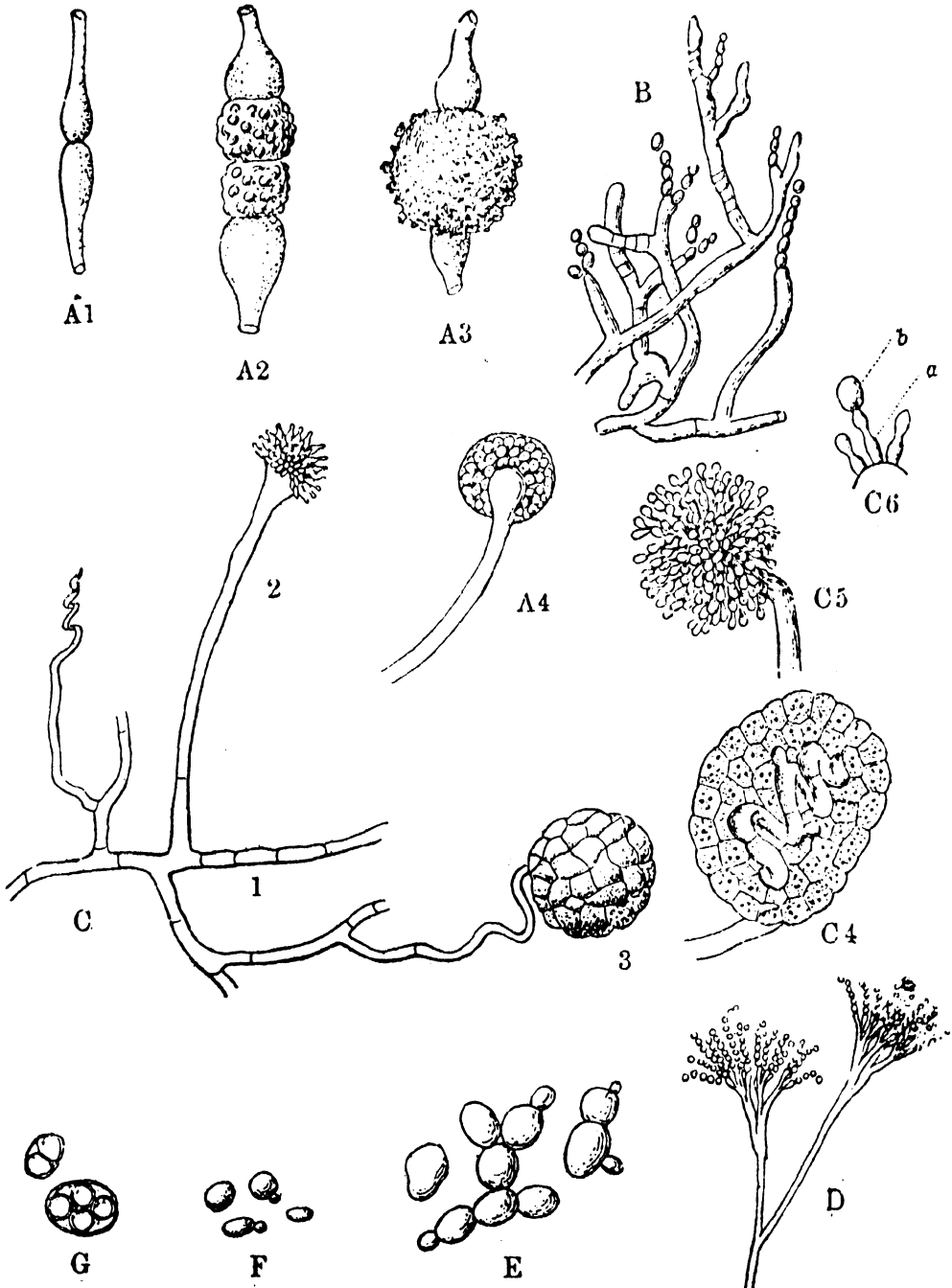


FIG. 194.—A, Mucor Mucedo; 1, 2, 3, stages in formation of a zygospore. 4, a sporangium containing spores. B, Oidium lactis. C, Aspergillus glaucus (De Bary). 1, mycelium. 2 and 5, conidiophore-bearing spores. 3, 4, a perithecium (4 contains rudimentary asci). 6, piece of conidiophore; *a*, sterigma; *b*, spore. D, branched conidiophore of Penicillium glaucum bearing spores. E, F, Saccharomyces cerevisiæ, cells are budding. G, ditto, formation of endospores (after Hansen).

The more highly organised fungi, the Mycomycetes, differ from the Phycomycetes in that (1) their characteristic sporangia or conidiophores are *definite*; (2) the hyphæ are septate, with numerous cross partitions; (3) the sexual process, organs, and cells are so modified as to be more or less difficult of recognition. In any case, the Mycomycetes never, as an immediate result of a sexual fusion, produce a well-defined zygospore or oospore capable of developing into an independent vegetating fungus.

Two main series are recognisable in the Mycomycetes. In one series the sporangium has become definite in type, as it produces inside it a number of spores that is definite and constant to the species. The number of spores is usually eight, but a few species produce other multiples of two. This definite sporangium is termed an *ascus*, the spores are *ascospores*, and the group of fungi having asci is the *Ascomycetes*. In some of the Ascomycetes the asci are grouped together and form a kind of fruit-body (ascocarp), which, to give an example, is a closed spherical body in *Aspergillus* and *Penicillium* (*vide infra*). The production of asci is, at least often, preceded by a sexual fusion.

In the other series of Mycomycetes it is the conidiophore that has become definite in type, being constant and defined in form and numbers of conidia produced. The conidiophore usually bears four conidia or, in a few species, two or a multiple of two. Such a conidiophore is termed a *basidium*, and characterises the class *Basidiomycetes*, of which the common toadstools are examples.

The Smut- and Rust-fungi, *Ustilaginaceæ* and *Uredinaceæ*, are very simple Basidiomycetes, and among the salient features belonging to the members is their capacity to produce thick-walled resting-spores, which in germination give rise to a minute plant (a basidium) that buds off respectively indefinite and definite numbers of conidia.

In the Basidiomycetes the production of the basidium is a belated result of a sexual act in which the + (female) and — (male) nuclei do not fuse until long after the original protoplasmic fusion. In some species of the higher types an individual mycelium grown from one basidiospore will not give rise to the complete fungus with its mushroom-like fruit-body unless fusion takes place with another mycelium of similar origin: there are, then, + (female) and — (male) individuals. In other species one basidiospore can give rise to the complete fungus with its fruit-body, probably because the mycelium can produce + and — sexual nuclei.

The life-histories of some fungi placed in the groups already enumerated are incompletely known, yet certain characteristic stages are known, so that it is possible to refer these types more or less to their correct systematic position and class. For instance, the group *Chytridiinæ* includes a number of minute degraded parasitic fungi, some of which are even devoid of a mycelium, and clearly belongs to the Phycomycetes. But there still remain many kinds of fungi that are known only in their conidial stage, and the conidiophores are indefinite in type (not basidia). These imperfectly known fungi cannot be placed in their natural classes and have to be empirically grouped according to the arrangement and form of their conidiophores, structure and colour of their conidia, and so forth. They form the large unnatural group *Fungi Imperfecti*. Finally, there remain a few parasitic fungi known only in a sterile mycelial condition. As a number of pathogenic

› the *Fungi Imperfecti*, it may be pointed out that
›ns must be fulfilled if a fungus is to complete its
Many parasitic Uredinaceæ require two hosts. The
the sexual act in such a common fungus as *Mucor*
infra) demands a certain chemical composition of
medium. Very commonly a certain degree of desicca-
tes the sexual act and the production of fruit-bodies.
ıses the latter are not perfected in the absence of light.
is possible that some pathogenic Fungi Imperfecti are
s' ns, and will not yield diagnostic fruit-bodies until
h ght into contact with their opposites.
give examples of common non-pathogenic types.
ycetes : Mucor Mucedo (and other species of Mucor).—
m occurs on damp bread, horse dung, and other organic
ıata To the naked eye it appears as a white or smoky mould
›sed of fine filamentous usually non-septate hyphæ spreading
the substratum. Here and there arise erect hyphæ which
ıaturated atmosphere may attain a length of several inches,
which are very much shorter in ordinary air. Each erect hypha
nds in a spherical sporangium whose protoplasm is separated off
from that of the supporting hypha by a transverse wall, which
bulges greatly into the cavity of the sporangium and forms the
so-called columella. The protoplasm of the sporangium divides
into many masses, each of which acquires a cell-wall and is then
a spore. The spores escape by the rupture of the wall of the
sporangium. (The needle-like bodies often seen outside the wall
of the sporangium are crystals of calcium oxalate.) The less
frequent sexual method of reproduction and the formation of
the zygospore has already been described. The infrequency of the
sexual mode of reproduction is due partly to the fact that the
individual plants are sexually differentiated and might be termed
male and female. Zygospore and asexual spore alike germinate
to produce a new mycelium. In rich culture media or old cultures
the mycelium may become septate. Cultivated under water some
species (including *Chlamydomucor racemosus*) enter into an oidial
condition.

Ascomycetes: (1) **Aspergillus herbariorum** (=**A. niger**).—This,
with other varieties of the same group, is of frequent occurrence,
especially on dead vegetable matter. It grows readily on gelatin
and, to the naked eye, consists of a mass of filaments which micro-
scopically are seen to form a septate branching mycelium. Two
forms of reproduction occur, the variety depending largely on the
nutrition of the plant. The less common form is effected by means
of structures known as ascocarps, which owe their formation to a
sexual process. From a mycelial branch there arises a hypha
which becomes specially coiled and transversely septate at its end.
From the base of the lowest coil of the spiral two or three hyphæ
grow up towards its apex, where one of these fuses with the coiled
hypha and represents the male organ. The others by branching
copiously produce a mass of closely woven hyphæ forming a closed
wall to this structure, which is the ascocarp referred to. Within
it numerous asci arise as the ultimate ramifications of branches
given off by the central coiled hypha. Inside each ascus eight
ascospores are produced. Ultimately all the structures lying within
the ascocarp, save the spores, undergo disintegration, so that the

mature ascocarp consists of a small hollow sphei
lie the loose spores. These latter are ultimately
decay of the wall of the ascocarp and develop into n
The commonest method of reproduction is by the
spores in the form of conidia, which are clearly c
origin. A filament grows out, and at its terminatioi
swelling is formed on which a series of little finger-lil
called sterigmata are perched. At the free end of eac
rows of oval conidia are successively abstricted. F ch
on becoming free, can give rise to a new individual, 't
ascospore.

(2) **Penicillium crustaceum** (=**Penicillium glaucu**
perhaps a composite species, and is the most common o.
met with in bacteriological work. It is the common gree..
mould, and its extraordinary versatility and powers of resis
make its spores practically omnipresent. The mycelium is
that of the *Aspergillus*. Ascocarp formation takes place, but
commonest mode of reproduction is by the conidia. A filai
(the conidiophore) grows out, and at its end frays out into a pei
of finger-like branches. On the point of each of these a peg-lik
sterigma is developed. On the end of this a row of oval nidia
is successively cut off; these break off and can give rise to new
individuals.

(3) **Saccharomyces or Yeasts (Torula, Mycoderma).**—These organisms have been subjected to much investigation in consequence of their economic importance in brewing and baking. They occur in nature chiefly in connection with fruits, such as the grape, which contain fermentable sugars. They consist of round or oval cells, 3 to 5 μ in longest diameter, and under ordinary conditions reproduce themselves by budding, in which process a portion of the cell protrudes, increases in size, and finally becomes separated from the parent cell so as to form a new individual. In a number of other fungi belonging to the various groups, the conidium, when cultivated in a liquid, has the power of budding off conidia which behave in like manner; such fungi, therefore, have a yeast-like stage in their life-history. Under certain conditions of moisture and oxygen supply, endogenous sporulation occurs As the spores produced are definite in number—two in some species and four in others—the sporangium is an ascus and *Saccharomyces* is a degenerate ascomycete. In certain species (and possibly in all) conjugation of two yeast cells and fusion of their nuclei take place: this process may immediately precede the production of an ascus. While in yeasts generally the oval cell represents the vegetative unit, in certain species elongated tube-like bodies may be formed which suggest an attempt at hyphal formation. In *Saccharomyces mycoderma*, the vegetative cells are so elongated and linked as to form a kind of simplified mycelium.

Fungi imperfecti : Oospora lactis (Fres.) (=**Oidium lactis**).—This is a common fungus in sour milk and sour bread, and can easily be cultivated on gelatin, where the colonies consist of short and fine septate filaments radiating from a centre. Here and there the hyphæ are divided, especially at the ends, into short oval or cylindrical segments, termed oidia, which act as spores. No other method of reproduction is known.

Diseases produced by Fungi

Tinea and Favus.—In dealing with the common fungoid infections of the skin, it is only possible here to give a short account of the methods employed in the investigation and of the more common types of fungi isolated.

Methods.—For ordinary purposes of diagnosis it is usual to place the epidermic scales or hairs in a solution of 7 grms of potash in 100 c.c. of water (Adamson) or in liq. potassæ (B.P.), to heat for a few seconds and to examine under a cover-glass. For permanent stained preparations Sabouraud recommends that the fat should first be removed by means of chloroform from the material, which is then placed in formic acid and warmed for two or three minutes till the fluid boils. The acid is removed by washing in distilled water and the preparation stained for a minute with Sahli's blue, which has the following composition: distilled water, forty parts; saturated aqueous solution of methylene-blue, twenty-four parts; 5 per cent. solution of borax, sixteen parts. The preparation is then washed, dehydrated in absolute alcohol, cleared in xylol, and mounted in balsam.

The glucose and maltose media of Sabouraud (p. 61) constitute the best means of isolating skin fungi, as by these not only are the most characteristic growths obtained, but there is a certain degree of inhibition of the skin cocci. Where, as in Tinea circinata, there is a vesicular or pustular lesion, the contents are squeezed out and transferred with a platinum needle to the medium. If there is a skin scurf, the squames may be scraped off on to a sterile slide from which tubes may be inoculated. When hairs are to be dealt with, these may be picked out on to a sterile slide, their roots cut off with a hot needle and planted in the medium. In certain hair affections, especially in animals, the parasite is specially abundant in the aerial part of the hair, so that portions of this, as well as the radical, ought to be used. It is often advisable, especially in pustular conditions and in favus, to place the hair in absolute alcohol for two minutes, to allow to dry and then plant on the medium.

Microspora.—These are the small-spored ringworm parasites, and are responsible for a large proportion of the ringworms of the scalp occurring in children; they only occasionally cause affections of the other parts of the body. In the initial lesion in the epidermis a fine mycelium, 1–5 μ in diameter, composed of rectangular elements, may be observed. This mycelium penetrates into the hairs where they emerge from their sheaths, and grows up and down in them. When an infected hair is examined, it is found to be encased with a mass of spores which have the characters of an irregular mosaic, the elements being frequently crushed together in polygonal forms and showing no tendency to an arrangement in rows. These spores are about

2 μ in diameter, but in potash preparations may appear larger—up to 5 μ. According to Sabouraud, the appearance on the hair results from intra-capillary mycelial threads breaking out at numerous points on the surface and there undergoing irregular longitudinal and transverse splitting, to form the spores. The mycelium can be demonstrated by mounting the hair in 7 per cent. potash solution and disengaging the adherent and obscuring spores by gently rubbing the hair between the slide and the cover-glass. The species most commonly present is the *Microsporon audouini* (Fig. 195), and a number of allied species have been isolated in the dog, the cat, and the horse, and these are

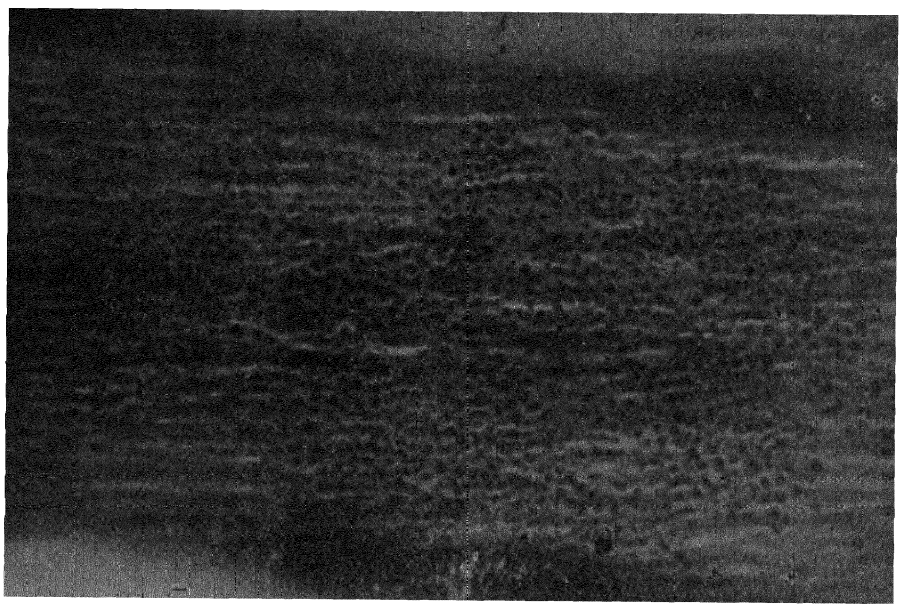


FIG. 195.[1]—Hair infected with Microsporon audouini. Photograph of unstained preparation. ×500.

of importance from the frequent infection of man from such animal sources. Other species, e.g. *M. velveticum*, *M. umbonatum*, and *M. tardum*, presenting cultural differences, have been observed in man.

Trichophyta.—These fungi, which constitute the large-spored ringworms, are associated with ringworm of the scalp, with the various manifestations found in the beard, and with the conditions occurring on the smooth parts of the body and in the nails. They are characterised by the fact that the mycelium, wherever observed—whether in epithelial squames, in pus, or within a

[1] For Figs. 195–201, 206, we are indebted to the kindness of Dr. R. Cranston Low.

hair—consists of chains of oval or rectangular spore-like bodies (Fig. 198). These in the largest forms are from 5–8 μ in diameter, but smaller forms approaching the size of the spores in microspora also exist. There is thus not the same differentiation between mycelium and spores seen in the microspora, nor does the irregular mosaic appearance of the spores in the latter come into evidence. There is, however, the same primary affection of the superficial epithelium, and in hairy parts the same invasion of the hair where it emerges from its sheath.

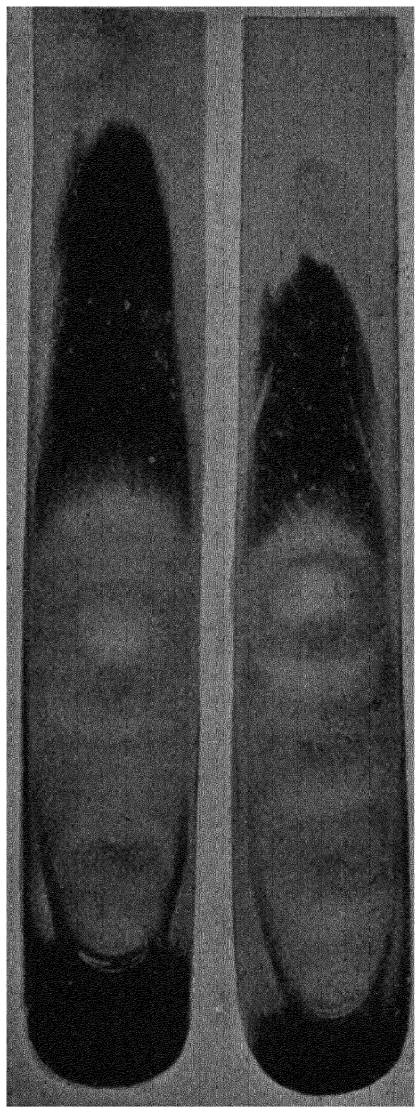
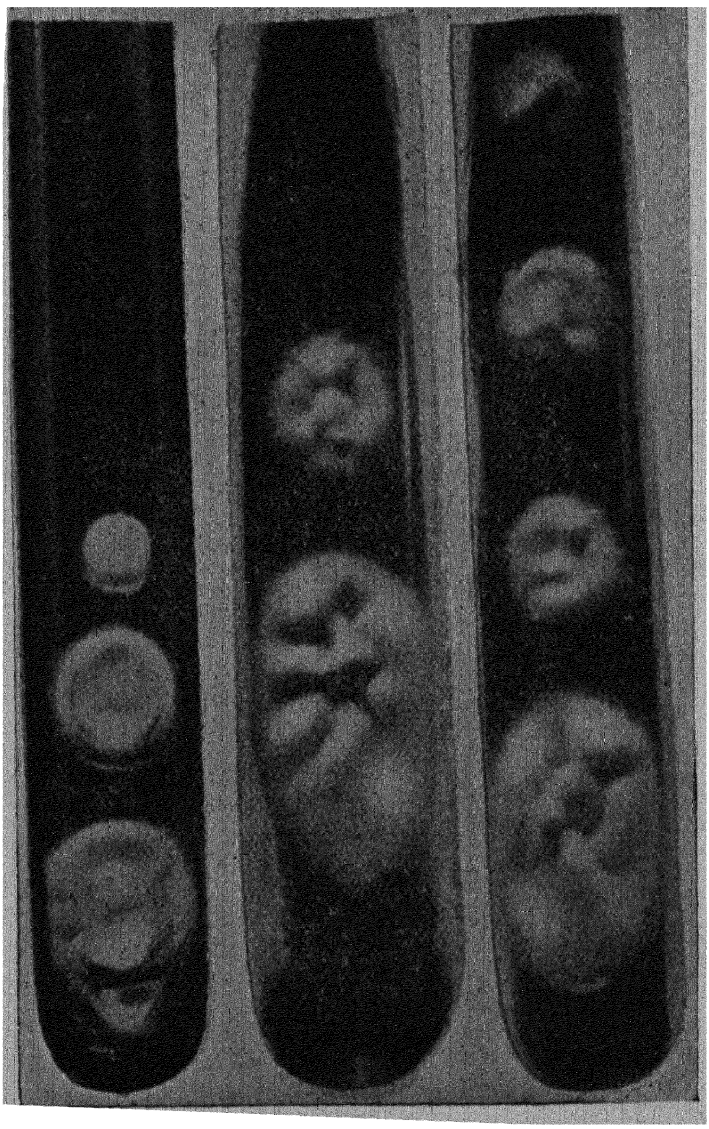


FIG. 196.—Microsporon audouini on Sabouraud's maltose agar.

In certain species there is a tendency for the parasite to invade the follicle by growing down between the hair and its sheath for a considerable period before the hair itself is invaded,—the so-called *Trichophyton ectothrix*. A great number of trichophyta presenting different cultural characteristics have been isolated. These are associated with difference in site of election and in method of spread in different parts of the body. There is evidence that certain varieties are more common in some countries than in others; for instance, in France *Trichophyton acuminatum* is the commonest, whereas in Scotland *Trichophyton crateriforme* (variety *flavum*) (Fig. 197 *a*) is the most frequent cause of large-spored ringworm of the scalp, and *Trichophyton rosaceum* (Fig. 197 *b*) of ringworm of the beard. In France another coloured variety—*Trichophyton violaceum*—is of common occurrence. Similar organisms have been described in the lower animals, such as the horse, calf, and dog, and the infection of man from such sources is relatively frequent.

The pathological lesions produced by the microspora and

trichophyta are similar, though those of the latter are the more severe. In each case there is primarily a premature detachment of epithelial squames with subjacent inflammation in the corium, frequently followed by a slight hyperkeratosis, especially



a *b* *c*

FIG. 197.—*a*, Trichophyton crateriforme. *b*, Trichophyton rosaceum. Sabouraud's medium.

marked around and within the hair sheaths. Follicular pustules are also common and in the most severe trichophytal cases a granulomatous condition (kerion) of the true skin, with relatively massive follicular suppuration, occurs.

Achoria.—These organisms are responsible for the various

clinical manifestations grouped under the name of favus, which affect both the hairy and smooth parts of the body. The characteristic of these is the development of round sulphur-yellow discs (*scutula*), each with a depression in the middle which in hairy parts often corresponds to the position of a hair follicle. These discs really consist of dense masses of fungoid growth (Fig. 201). The initial change is a vigorous invasion of the epithelial squames, sometimes accompanied by an intra-epider-

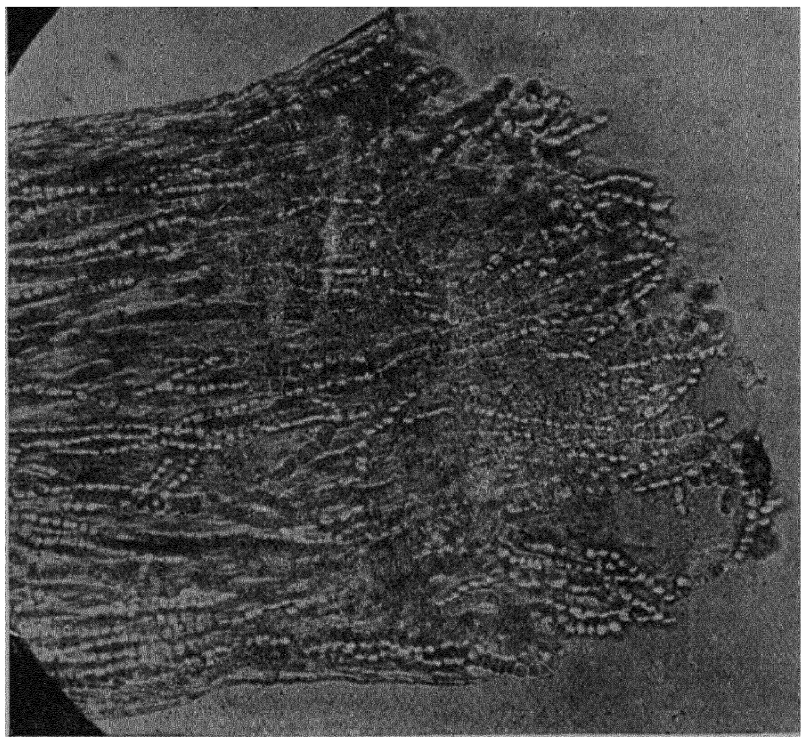


Fig. 198.—Hair infected with large-spored ringworm. Photograph of unstained preparation. ×500.

Note.—The sizes of the spores in Figs. 195 and 198 are not comparable, as in photographs of such thick preparations it is impossible to focus the outlines sharply.

mic, very often circumpilary, suppuration. As in the conditions previously described, the hair becomes invaded, the shaft being especially affected, but the hair infection is of subsidiary importance. The feature of the affection is the destruction of skin structures (*e.g.* hair follicles), this leading, when recovery takes place, to the affected part assuming a cicatricial character. Sometimes a granulomatous affection of the skin is observed, which may be due to secondary infections. Preparations from the crusts (Fig. 201) show the presence of spores and mycelial threads,

whose elements vary much in size and shape, but which are generally larger than those of the trichophyta. The affection of the hairs is severe, and the track of the mycelium is often marked by the presence of comparatively large air channels (Fig. 199). The commonest fungus present is the *Achorion schönleinii* (Fig. 200 *a*), but a great number of varieties occur; these can be readily cultivated on Sabouraud's media. The lower animals (fowl, mouse, dog, cat (Fig. 200 *c*)) are often affected.

Of the less common skin fungi, *Epidermophyton inguinale*, found in eczema marginatum, deserves mention. In preparations of the epithelial scales the organism presents itself in complex undulating

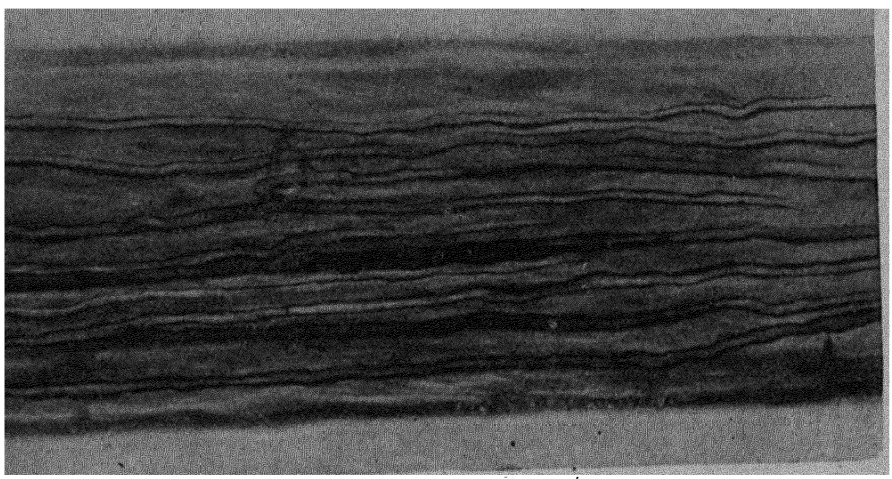


FIG. 199.—Favus hair showing air channels left by mycelium. ×300.

threads consisting of short elements 4 to 5 μ broad and 4 to 12 μ long. Its characters mark it off from the organisms described. The hairs in the diseased area remain unaffected, but the organism is closely allied to the trichophyta, though it is not so easily cultured. Infection experiments with cultures have hitherto failed.

It is unnecessary to describe in detail the characters presented in cultures by the three groups of parasitic skin fungi, and we need only mention certain commonly occurring characters. In all there is a free production of a septate mycelium, and usually, by a lateral budding from the hyphæ or by the breaking up of the protoplasm of the thread, there is the formation of bodies resembling those described as spores which occur in affected tissues. This spore formation often shows a tendency to occur specially at the termination of filaments. Sometimes in the course of a filament an element enlarges and from it new mycelia sprout, the whole resembling chlamydospore formation.

Sometimes, especially in the microspora and the achoria, large fusiform elements divided by transverse septa are observed, which suggest conidia formation. Curious spiral elements whose significance is unknown, are also frequently seen.

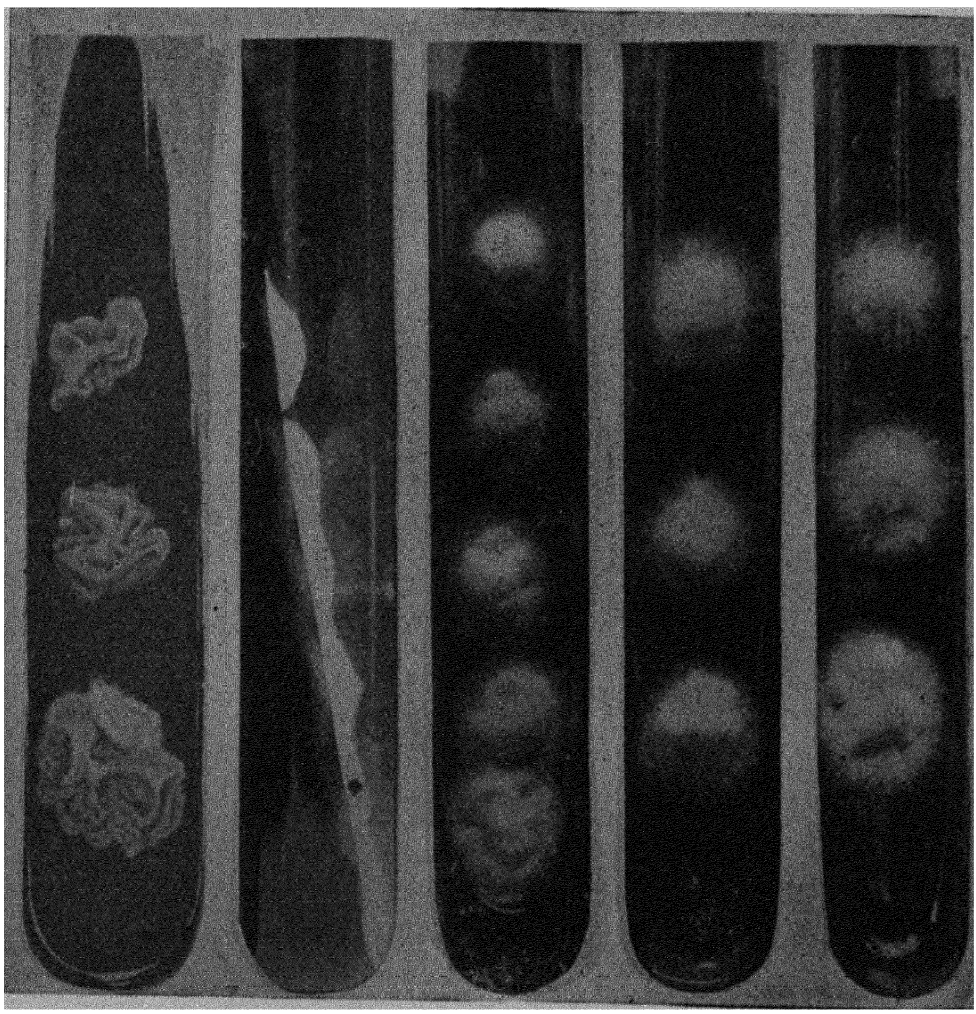


FIG. 200.—*a*, Photograph of drawing of Achorion schönleinii on Sabouraud's maltose agar. *c*, Photographs of cultures of Achorion quinckeanum. (The central culture of *c* was isolated from a cat, and the two side tubes from a man infected from it.) *b*, Side view to show elevation of growth.

For a full description of the naked-eye characters of the various ringworm and favus fungi, the reader must be referred to such works as those of Sabouraud. The characters vary very much with the medium employed, and hence in any comparative study it is of great importance that the same medium should be

used; and it is even necessary that a large bulk of a medium should be made up at once so as to be available for an extended study.

On Sabouraud's media most of the fungi at the commencement of their growth appear as white fluffy or felted button-like colonies on the surface, and as growth proceeds differentiating characters emerge. Thus the organism may tend to spread in a fairly thin layer over the medium and sometimes there is an appearance of successive concentric rings of growth; on the other

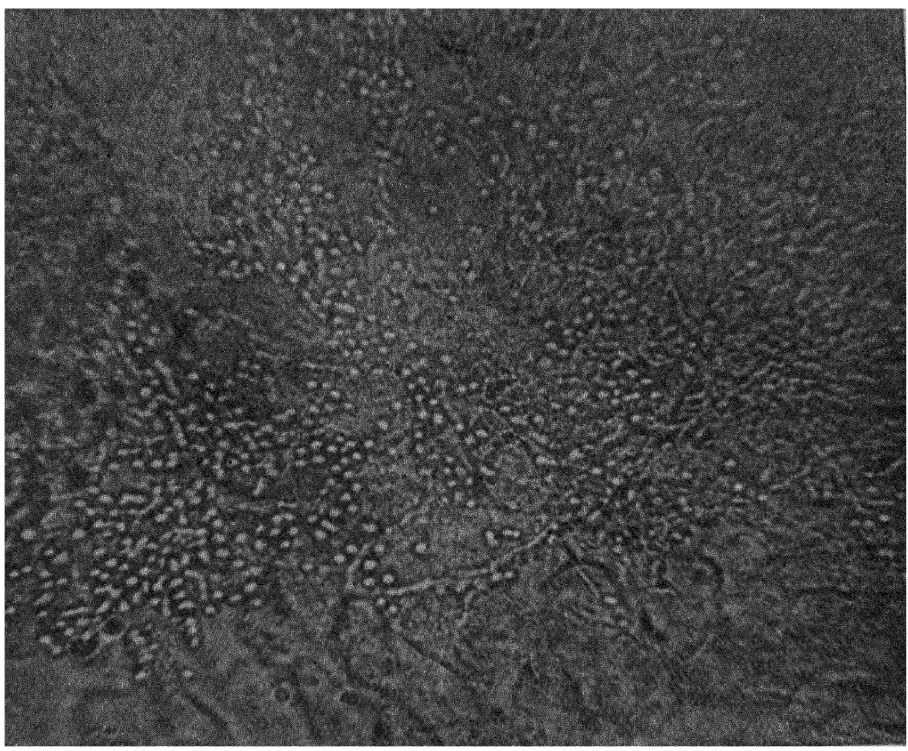


FIG. 201.—Photograph of drawing of scraping from favus scutula, showing spores and mycelium. Unstained. ×250.

hand, the colony may be heaped up in the centre as a projecting knob, or there may be a central depression round which the heaping up occurs. Sometimes there are ridges or folds radiating from the centre of the colony, presenting a geometrical arrangement or having an irregularly convoluted appearance. The surface may have a woolly character or may give the impression of being covered with fine powder. Sometimes the surface formation is moist and slimy-looking. These appearances are exemplified in Figs. 196, 197, 200. When colour is produced it develops with age. An important point is the occur-

rence of pleomorphism. Thus a sub-culture frequently presents characters different from those of the parent growth, or on a coloured colony colourless points may appear which may maintain the non-pigmented character when sub-cultured. The evidence at present is that these are cases of true pleomorphism and are not due to contaminations. On media presenting large surfaces the colonies assume a correspondingly large size and growth usually goes on until the medium is exhausted.

Generalised Lesions.—Within recent years it has come to be recognised that the conditions just described are not always merely local lesions, as was formerly supposed, but that occasionally in both types of ringworm (especially in deep-seated lesions due to trichophyta) and in favus, generalised skin eruptions occur. These are known as trichophytides, microsporides, and favides respectively. The eruptions are of various kinds—papular, vesicular, pustular, etc., and their appearance is accompanied by polymorpho-nuclear leucocytosis, fever, etc.; they are manifestly hæmatogenous in origin. In a certain number of cases the parasite of the original infection has been found in the secondary lesions, and in a few instances has actually been cultivated from the blood. The view has, accordingly, gained acceptance that the fungi in question may occasionally become disseminated by the blood stream and arrested in the blood vessels of the cutis, where their toxins act on an allergic skin. In many instances, however, there is no evidence of the presence of the parasite, and the rash is of such a nature, for example scarlatiniform, that it is hardly possible that it is produced in this way. It is, apparently, of the nature of a supersensitive reaction, due to the toxins of the parasite, and it is to be noted that allergy of the skin has been demonstrated in such cases. Bloch, for example, produced scarlatiniform and lichenoid eruptions by intravenous injection of trichophytin in cases of kerion. At present it is not possible to say how far the actual presence of the organisms is concerned in the production of generalised eruptions.

Immunity and Supersensitiveness.—A large amount of work has been done on these subjects in recent times, but many of the results are conflicting; the following is a short summary of what seems to be established. General reactive phenomena evidenced by supersensitiveness and the presence of antibodies in the blood are met with in the fungus infections, but only when there is considerable involvement of the connective tissues, *e.g.*, in deep-seated ringworm, sporotrichosis, hemisporosis, etc. The reactions are analogous to those which are recognised as

occurring in granulomatous infections, *e.g.* tubercle, and are not attended by an immunity to the invading parasite. Evidences of supersensitiveness or allergy are obtained by injection of extracts of the parasite. For example, in deep-seated ringworm such an extract (trichophytin) produces a local, a focal, and a general reaction, the last being attended by pyrexia, etc. (cf. tubercle, p. 335). When, however, the parasite has merely a superficial distribution, such reactions have not been found. The presence of antibodies such as agglutinins, complement fixation bodies, etc., has been observed in infections with much tissue change, such as those mentioned above. It is to be noted, however, that such reactions are not strictly specific, as they are given, though often in less degree, with other fungi than the invading organism, the results indicating a certain degree of common antigenic structure of the fungi. Extracts of the parasites have been used in treatment, and success has been recorded in the case of the ringworm infections. Certain of the ringworm and favus parasites produce in some of the laboratory animals infections which undergo spontaneous cure, and this has been found to be attended by an immunity of the whole skin. In the human subject, however, local immunity does not follow or is of very short duration ; ringworm, for example, may recur in a part previously the seat of the disease.[1]

Thrush (German, *Spoor* ; French, *Muguet*).—This condition, which is most common in children, chiefly affects the tongue and fauces, and may extend into the œsophagus. It is characterised by white patches largely composed of fungoid growth, which cause catarrh of the subjacent epithelium and slight erythema. A similar condition may occur in the vagina, and a few cases of generalised affection with abscesses or tubercle-like lesions in the solid organs, *e.g.* the lungs, have been recorded. The organism closely resembles the *Oospora* (*Oidium*) *lactis* (*vide* p. 712), very frequently found in milk, and has been called *Oidium albicans* or *Monilia candida*. It occurs in two chief varieties—a large-spored and a small-spored form, the former being the more frequent. Both in the tissues and in cultures the chief elements are double-contoured, septate mycelial threads—the elements being of varying sizes,—and round or oval spores (in the large-spored type 5–6 μ long and 4 μ broad). The fungus grows readily on artificial media, especially those containing beerwort (p. 61), and while some varieties liquefy gelatin, others do not. In the case of the latter, the superficial

[1] For details, the reader may be referred to *Anaphylaxis and Sensitisation*, by R. Cranston Low, Edinburgh, 1924.

colonies on gelatin are granular with peripheral feathery extensions, while the deep colonies are rounder and more circumscribed. The colour is white or slightly red, and the cultures have a sourish alcoholic smell due to the production of aldehyde, alcohol, and acetic acid ; glucose, lævulose, and maltose are slowly fermented, but the fermentation reactions vary in different species of moniliæ. On ordinary media, mycelium and spore production are seen, the former being especially marked in deep colonies. Formation of chlamydospores is also stated to occur, and from such elements on a mycelium, free conidia formation takes place.

Aspergillosis.—In 1856, Virchow recorded several cases of affection of the lungs by aspergilli, and a number of similar cases have since been described ; usually there has existed some other disease in the body, and frequently the lung has also been the site of tuberculosis. The appearances presented are those of small grey nodules composed of necrotic material and leucocytes, which break down to form cavities associated with areas of broncho-pneumonia, and frequently also with fairly widespread odourless necrosis of the lung. Masses of fructifying mycelia are present in the cavities and extend into surrounding bronchioles and air cells. The condition has usually been discovered *post mortem*, but in certain cases the fungus has been observed in the sputum during life, and it is probable that a lung condition of this kind can be recovered from. A similar affection occurs in birds. It is probable that infection arises from inhalation. The variety of organism chiefly present is the *Aspergillus fumigatus* (cf. p. 711), which on artificial media gives a greenish-blue colour resembling that of the *Penicillium crustaceum*. Its optimum temperature is that of blood heat.

Infections with aspergilli also occur in the external ear in the form of chronic pustular conditions, and aspergillary colonies are also from time to time met with on abrasions of the cornea.

Sporotrichosis.—In 1898, Schenk, in America, described a case of chronic subcutaneous abscesses associated with a fungus belonging to the *Sporotricha*, and during recent years the organism has been isolated from a great many granulomatous conditions occurring in various parts of the world. Most of the cases have been characterised by somewhat heteromorphic and indolent granulomatous lesions in the skin, resembling those of tuberculosis and syphilis. The initial lesion is at the site of some slight abrasion, and it is followed by a succession of granulomata, usually small, whose distribution indicates a lymphatic spread. There is little tendency to spontaneous cure. Apart

from the skin, cases have been recorded of lesions in the pharynx, larynx, muscle, bone, and synovial membrane ; and both in man and in animals (dogs, rats) generalised infections of the serous cavities and solid organs have been observed. The lesions are of a diffuse granulomatous character, and at first consist chiefly of young connective-tissue elements with plasma cells and lymphocytes. Later, diffuse degeneration and necrosis occur and also leucocytic emigration with the formation of abscesses,

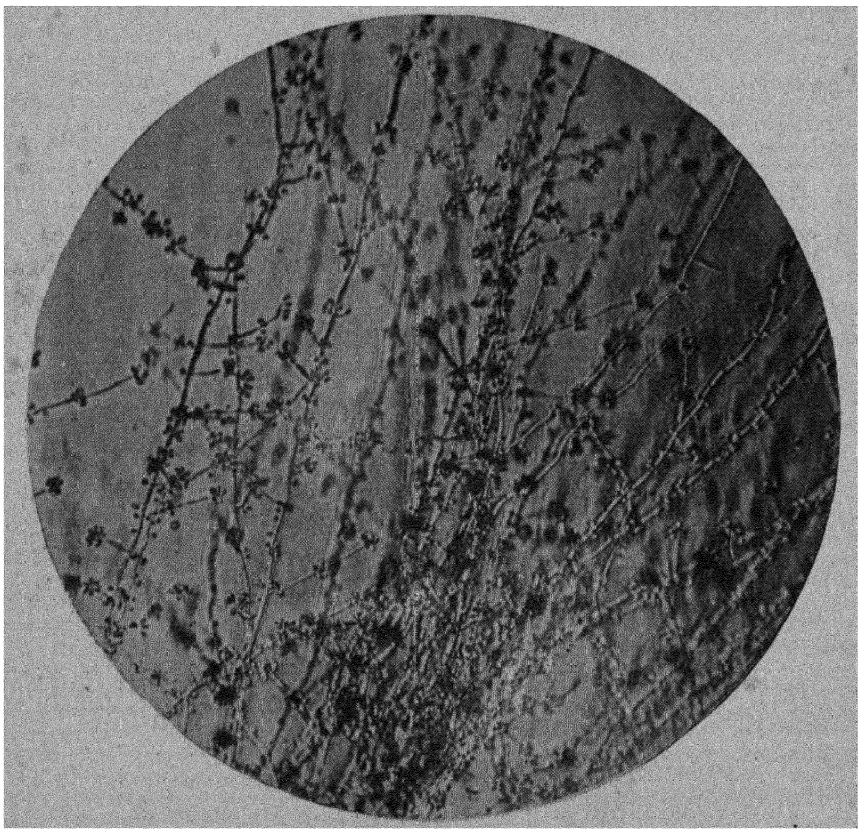


FIG. 202.—Edge of living colony of Sporotrichon beurmanni on agar hanging-drop, five days at 22° C. ×200.

at first of microscopic size. When the skin is involved, ulceration results. In certain cases abscess formation is more marked. No generalised skin eruptions, such as occur in tinea and in favus, have been observed in sporotrichosis. The serum of patients suffering from the disease has been found to contain antibodies ; but the reactions are not specific, as they are given also with other fungi.

Direct examination of the pus may reveal the presence of oval, highly refractile spores, 3–4 μ long and 1·6–3 μ broad, and these may be demonstrated both free and in the granulomatous

cells, in films and sections stained by ordinary aniline dyes ; they are Gram-positive. Mycelial formation does not occur in the tissues, except occasionally in the most superficial parts of an ulcerating lesion. If a drop of pus be placed on the glass of an agar tube just above the condensation water, the sprouting of mycelium from the spores may be directly observed with the microscope. The organism, which is generally known as the *Sporotrichon beurmanni*, grows readily on any ordinary medium (gelatin, agar, potato), but is best studied on Sabouraud's medium. Two sets of media should be inoculated—one incubated at 37° C. and the other at room temperature. On the latter, after about forty-eight hours, somewhat fluffy, snowflake-like, white points appear which gradually become brown, and when growing in mass present a heaped-up convoluted growth. The morphology of the organism is best studied in hanging-drop preparations made with agar. From a spore a mycelial thread about 1 μ in thickness, irregularly septate, and often containing fine granules, sprouts off. Lateral branches arise and fresh spore-formation is soon observed. These usually develop in whorls round a filament (Fig. 202), but sometimes the process occurs all along a filament. Sometimes, in the course of a filament, large circular elements, 5–6 μ in diameter, resembling the zygospores of Mucoraceæ are seen, and these sometimes contain groups of spore-like bodies. The free growth of the organism depends on conditions of moisture and temperature, and where these are unfavourable, instead of mycelial formation being observed, the spores may enlarge to three or four times their ordinary size and then give off circles of fresh spores (Fig. 203).

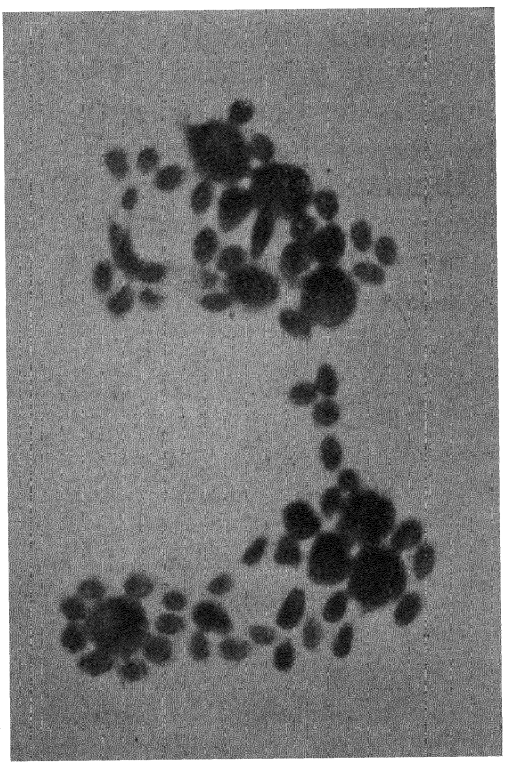


FIG. 203.—Film from agar culture of Sporotrichon beurmanni grown at 37° C. for ten days. Gram's stain. ×1025. Note large circular bodies with spores sprouting off; also a few sausage-shaped elements.

Under a low power of the microscope, mycelial colonies have a stellate appearance with a very freely spiked edge. The organism manifests considerable vitality under saprophytic conditions, and is said to have a fairly widespread distribution. Cultures are pathogenic when injected subcutaneously in mice, rats, dogs, etc., granulomatous lesions identical with those of the natural disease being produced.

Sporotrichosis in man has probably often been confused with the manifestations of syphilis, as the condition readily yields to the administration of potassium iodide. In horses, certain cases presenting the characters of epizootic lymphangitis have been found to be associated with an organism indistinguishable from the *Sporotrichon beurmanni*.

Hemisporosis.—This is a fungus infection first described by Gougerot and Caraven who obtained from the lesions pure cultures of a parasite which was identified as the *Hemispora stellata* (Vuillemin). Since then a considerable number of cases have been reported, especially from Italy. Like sporotrichosis, it is characterised by the formation of granulomatous growths which may resemble syphilitic or tubercular lesions, and which may occasionally undergo suppurative softening. There may be a single lesion, or there may be multiple lesions, *e.g.* nodules in the skin; occasionally the bones are affected. Gougerot and Caraven found that the serum of the patient gave agglutination and complement fixation reactions with the parasite. These, however, are not strictly specific, as the reactions may be obtained with other fungi. They found that the organism had a similar pathogenic action in the rabbit.

The name *Hemispora stellata* was applied to the parasite owing to the star-like arrangement of the conidiophores on the surface of the mycelial growth, as seen under a low magnification. It is a fairly common saprophyte in nature, occurring in the dust of rooms, etc. On culture media it forms an irregular whitish layer of indefinite extent. Certain of the mycelial threads become somewhat swollen at their free extremity, forming protoconidia, and these become segmented into 3–8 subspherical deuteroconidia, measuring 2·5 $\mu \times 3{\cdot}5$ μ which constitute spores. It may be difficult to detect the organism by microscopic examination of the tissues, while cultures may be readily obtained on various media.

Blastomycosis.—In pathological literature there are recorded a very large number of usually isolated cases presenting the characters of granulomata or of chronic suppurations, in connection with which the presence of yeast-like bodies has

been observed, and from which cultures of these have been obtained. The relation of the organisms isolated to the known types of fungi is largely undetermined. In the tissues the organisms usually appear as single double-contoured cells which multiply by budding or by a process resembling endogenous sporulation; while in artificial cultures, although similar appearances may be seen, a tendency to mycelium formation is frequently observed. The term "blastomyces," which may be taken as synonymous with yeast, finds no place nor has it any specific significance in modern descriptive fungology, for in vastly differing species yeast-like elements occur representing stages in development. From their tendency to produce mycelia, the organisms concerned in the so-called blastomycosis probably approach most nearly to the oidia (oospora), so that *oidiomycosis* might be a more scientific denomination of the diseases in question.

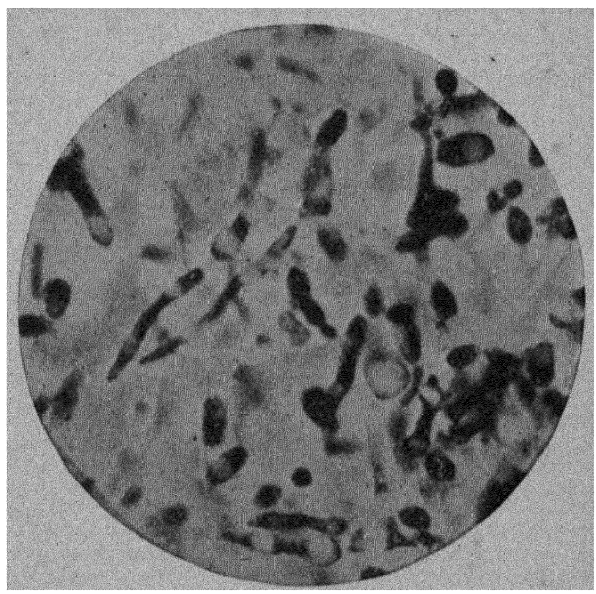


FIG. 204.—Growth of blastomyces in kidney of rabbit infected from human case (see text). ×1000.

While organisms of this group have been isolated from many conditions, for example, rabies and malignant tumours, in which there is no evidence that they play an etiological rôle, there is no doubt that they can multiply and cause pathological changes in the animal body. An example of this is seen in Fig. 204, taken from the kidney of a rabbit which was inoculated subcutaneously with an organism isolated from the sputum of a human case of obscure granuloma of the lung, associated with a suppurative condition in the kidney and the presence of similar organisms in the urine. In this case the appearances of the organisms in the tissues corresponded to those seen in cultures. In the conditions about to be described, and of which we have had no personal experience, difficulties present themselves in that the supposed causal agent appears in the tissues in the form of a peculiar round double-contoured cell (Fig. 205) not exactly

reproducible in artificial cultures. The appearances of these cells are rather suggestive of protozoal characteristics and, while a mycelial formation is stated to have been observed to originate from them, their nature is still doubtful. They have been observed in two disease manifestations which we may now describe. The first of these is the blastomycotic dermatitis, widely studied in America, and especially in Chicago. The disease may arise in any part of the skin and frequently follows a slight wound. The development of a sluggish papule, becoming pustular and ulcerative, is followed by a slowly extending granular and papillomatous appearance, with irregularly distributed pustule formation, and surrounded by a reddened areola containing numerous miliary abscesses. Areas of this kind, several inches in diameter, may slowly develop. These may heal at one margin and extend widely at another. The process may go on for years, and various, it may be distant, parts of the skin may become successively affected. In the great majority of cases no general disturbance occurs. Microscopically, in the fully advanced stage, the picture is that of an irregular epithelial proliferation and hyperkeratosis with superficial papillomatous excrescences, and more deeply of a similar irregular and free epithelial proliferation taking place in a granulomatous condition of the cutis. Special features are the development of minute pustules, partly intra-epithelial, partly in the corium, and the formation of giant cells. In the pus, organisms presently to be described are found. The disease is of chronic nature and is restricted to the skin.

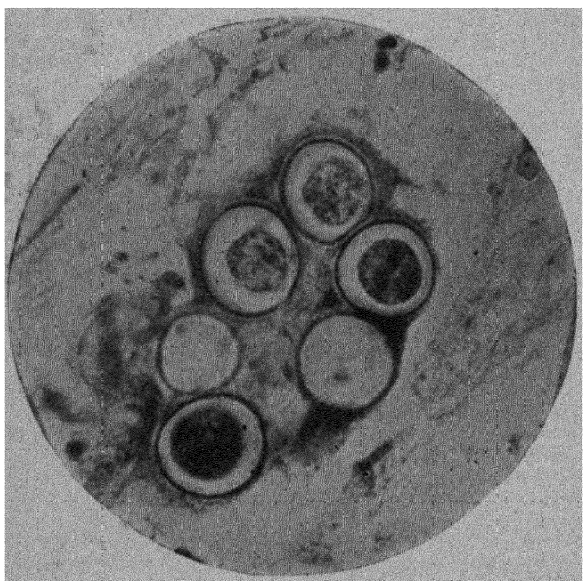


FIG. 205.—Double-contoured bodies in tissues from one of Rixford and Gilchrist's cases. ×500.

A closely allied condition is that described by Wernicke in South America and by Rixford and Gilchrist in California. In the first described cases attention was directed to the occurrence of suppurative conditions in the lungs. A skin lesion also

occurs, characterised by subcutaneous abscesses or granulomata leading to ulceration with epithelial hyperplasia. This may be the primary manifestation of the disease, but the internal organs become affected with chronic suppurative processes or granulomata, and death occurs. Cases belonging to the same class are also recorded where subcutaneous nodules, consisting of myxomatous connective tissue, have been observed, associated with the occurrence of suppurations in the internal organs. The cases of generalised infection were at first attributed to protozoa. The direct observation, under the microscope, of the growth of a mycelium from the protozoon-like body is the evidence adduced for the fungoid nature of the organism and led to its being designated *Oidium coccidioides*.



FIG. 206.—Microsporon furfur; scraping from skin. Gram's stain. ×1000.

The organisms isolated from these varying lesions are evidently all closely allied. In blastomycotic dermatitis they are present chiefly in the abscesses in the corium, and can be demonstrated by mounting the pus in 30 per cent. caustic potash solution. They are spherical in form, 8 to 10 μ in diameter, and appear singly, in pairs, or less frequently in larger groups (Fig. 205). There is a central protoplasm without distinct nucleus, separated by a delicate membrane from a surrounding clear space, and the whole is enclosed in a highly refractile, double-contoured capsule. Budding is frequently seen. The organisms stain with hæmatoxylin and with basic dyes and are Gram-positive, the reaction of the capsule being variable. The organisms present in the generalised infections are much more numerous in the tissues and attain the size of 35 μ. In these there are appearances in the protoplasm which suggest endogenous sporulation. The facility with which the fungi have been cultivated varies in different cases, but growth can usually be readily obtained at room temperature or at 37° C. on ordinary media, but preferably on Sabouraud's maltose medium,

especially when this is made slightly acid. Growth appears in from two to seven days, and the characteristics vary. In some cases moist, paste-like colonies develop, in others the surface appears crumpled, and sometimes it is dry and powdery. These differences are associated with differences in the degree of mycelial formation, in the extent of the ingrowth of the organism into the medium, and in the presence or absence of aerial conidia. The effects of the different varieties differ. Glucose and maltose are usually fermented ; gelatin is ordinarily not liquefied ; and indol formation is uncommon. In cultures, the budding seen in the tissues is also observed, and there is a varying amount of formation of segmented and branching hyphæ, this in certain cases being particularly well marked and giving rise to a definite mycelium. Somewhat slender aerial hyphæ sometimes occur which may form lateral spherical conidia, and sometimes terminal bodies resembling ascospores. The elements in cultures resembling those seen in the tissues frequently also possess a double-contoured capsule.

A considerable number of the organisms isolated are pathogenic for animals. Abscesses follow subcutaneous inoculation in guinea-pigs, rabbits, and mice, and death may result. Intravenous injection may result in a fatal pulmonary infection ; intraperitoneal infection is often without result.

Microsporon Furfur.

This is the organism associated with *pityriasis versicolor*. The condition, which is very widespread all over the world, occurring often in phthisical patients, is not looked upon as a disease of the skin, but is due to the saprophytic growth of the microsporon on the skin surface. The organism can be demonstrated in scrapings from the lesion, either examined in potash solution or in films stained by, for example, Gram's method. The organism consists of an irregularly contoured crumpled mycelium in segments 7–13 μ long and 3–4 μ broad. Associated with this, there are irregular groups of double-contoured spore-like bodies 4–7 μ in diameter (Fig. 206). Nothing further is known regarding the organism, as most attempts at cultivation have had a negative result, and even where cultures are said to have been obtained it has been impossible to secure continued growth.

APPENDIX

THE BACTERIOLOGY OF AIR, SOIL, WATER, SEWAGE, AND MILK. ANTISEPTICS

As this work deals essentially with bacteriology in relation to pathology and clinical medicine and surgery, its scope does not include a full account of the applications of the science to practical sanitation. It is convenient, however, to give an outline of some of the methods employed in sanitary work and to indicate the chief results obtained.

Air

Very little information of value can be obtained from the examination of the air, but the following are the chief methods used, along with the results obtained.

Methods of Examination.—The methods employed vary with the objects in view. If it be sought to compare the relative richness of different atmospheres in organisms, and if the atmospheres in question be fairly quiescent, then it is sufficient to expose agar plates for definite times in the rooms to be examined. In each case one plate is incubated at 37° C. and one at about 22° C. Bacteria, or the particles of dust carrying them, fall on the plates, and from the number of colonies which develop a comparative estimate of the richness of the air in bacteria can be obtained. Petri stated that in five minutes the bacteria present in 10 litres of air are deposited on 100 square centimetres of a plate.

More complete results are available when some method is employed by which the bacteria in a given quantity of air are examined. Thus such a quantity of air may be bubbled by an aspirator through sterile water, and measured amounts of the fluid may be plated in nutrient agar. Of the more formal apparatus the following may be described :

Petri's Sand-Filter Method.—A glass tube open at both ends, and about 3½ inches long and half an inch wide, is taken, and in its centre is placed a transverse diaphragm of very fine iron gauze (Fig. 207, *e*) ; on each side of this is placed some fine quartz sand which has been burned, well washed, and dried to remove all impurities, and this is kept in position by cotton plugs. The whole is sterilised by dry heat. One plug is removed, and a sterile rubber

cork, *c*, inserted, through which a tube, *d*, passes to an exhausting apparatus. The tube is then clamped in an upright position in the atmosphere to be examined, with the remaining plug, *f*, uppermost. The latter is removed and the air sucked through. Difficulty may be experienced from the resistance of the sand if quick filtration be attempted. The best means to adopt is to use an air-pump—the amount of air drawn per stroke of which is accurately known—and to have a manometer (as in Fig. 31) interposed between the tube and the pump. Between each two strokes of the air-pump the mercury is allowed to return to zero. After the required amount of air has passed, the sand *a* is removed; plate cultures are then made from it, and when growth has occurred the colonies are enumerated; the sand *b* is similarly treated, and acts as a control.

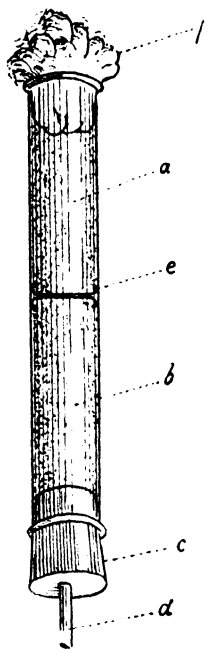


FIG. 207.—Petri's sand-filter.

When it is necessary to examine air for particular organisms, special methods must often be adopted. Thus, in the case of the suspected presence of tubercle bacilli, a given quantity of air is drawn through a small quantity of bouillon and then injected into a guinea-pig.

Comparatively little information bearing on the harmfulness of the air is obtainable by the mere enumeration of the living organisms present, for under certain conditions the number may be increased by the presence of many bacteria of a purely non-pathogenic character. The organisms found in the air belong to two groups—firstly, a great variety of bacteria, *e.g.* sporing bacteria of the B. subtilis group (*q.v.*), staphylococci of the albus type, chromogenic cocci, such as sarcina lutea; secondly, yeasts and the spores of moulds. With regard to moulds, the organisms consist of felted masses of hyphæ, from which are thrust into the air special filaments, and in connection with these the spores are formed. By currents of air these latter can easily be detached, and may float about in a free condition. With the bacteria, on the other hand, the case is different. These organisms are only present in the air when detached in dust from some dry substrate, or when introduced into the atmosphere from the exhalations of man and animals. The entrance of bacteria into the air, therefore, is associated with conditions which favour the distribution of dust and the presence of secretion droplets from the respiratory passages, etc. The presence of dust, in particular, would add a large number of bacteria to the atmosphere, and this is the case with the air in many industrial conditions, where the bacteria, though numerous, may be quite

innocuous. Great numbers of bacteria thus may not indicate any condition likely to injure health. On the other hand, there is no doubt that disease germs can be disseminated by means of the air. The possibility of this has been shown experimentally by contaminating the mouth with the B. prodigiosus, which is easily recognised by its brilliantly coloured colonies on culture medium, and then studying its subsequent distribution. The actions of coughing, sneezing, speaking, and even of deep breathing, distribute, often to a considerable distance, minute droplets of secretions from the mouth, throat, and nose, and these may float in the air for a considerable time. Even five hours after an atmosphere has been thus infected, evidence may be found of bacteria still floating free, though the majority have settled by this time. Examples of diseases in which infection can take place in this way are diphtheria, influenza, pneumonia, plague of the pneumonic type, and phthisis. In the case of phthisis, the deposition of tubercle bacilli has been demonstrated on cover-glasses held before the mouths of patients while talking, and animals made to breathe directly in front of such patients have become infected with tuberculosis.

With regard to infection by dust, a most important factor is whether or not the infecting agent can preserve its vitality in a dry condition. In the case of a sporing organism such as the anthrax bacillus, vitality is preserved for long periods of time, and great resistance to drying is also possessed by the tubercle bacillus. The aerial transmission of anthrax spores by dust and filaments from infected wool, *e.g.* in wool factories, has been well recognised (*vide* p. 390), and it is also well known that tubercle bacilli may be carried by dust. But apart from such cases there is little doubt that air infection is usually associated with the transport of secretion droplets, and is thus confined to a limited area around a sick person.

Soil

The investigation of the bacteria which may be found in the soil is undertaken from various points of view. Information may be desired as to the change its composition undergoes by a bacterial action, the result of which may be an increase in fertility and thus in economic value. Under this head may be grouped inquiries relating to the bacteria which convert ammonia and its salts into nitrates and nitrites, and to the organisms concerned in the fixation of the free nitrogen of the air. The discussion of the questions involved in such inquiries

is outside the scope of the present chapter, which is more concerned with the relation of the bacteriology of the soil to questions of public health. So far as this narrower view is concerned, soil bacteria are chiefly of importance in view of the fact that they can be washed out of the soil into water supplies. An important aspect of this question is the significance of certain bacteriological appearances in a water in relation to the soil from which it has come or over which it has flowed. In this country these questions have been chiefly investigated by Houston.

Methods of Examination.—For examination of surface soil or soil near the surface, Houston recommends tin troughs 10 inches by 3 inches, and pointed at one extremity, to be wrapped in layers of paper and sterilised by dry heat. If several of these be provided, then the soil can be well rubbed up and a sample secured and placed in a sterile test-tube for examination as soon as convenient after collection. If samples are to be taken at some depth beneath the surface, then a special instrument, of which many varieties have been devised, must be used. The general form of these is that of a large gimlet-like instrument stoutly made of steel. Just above the point of the instrument the shaft has in it a hollow chamber, and a sliding lateral door in this can be opened and shut by a mechanism controlled at the handle. The chamber being sterilised and closed, the instrument is bored to the required depth, the door is drawn back, and by varying devices the chamber is filled with earth; the door is re-closed and the instrument withdrawn.

In any soil the two important lines of inquiry are, first, as to the total number of organisms (usually reckoned per gram of the fresh sample); and secondly, as to the varieties of organisms present. The number of organisms present in a soil is often, however, so enormous that it is convenient to submit only a fraction of a gram to examination. The method employed is to weigh the tube containing the soil, shake out an amount of about the size of a bean into a litre of distilled water, and re-weigh the tube. The amount placed in the water is distributed as thoroughly as possible by shaking, and, if necessary, by rubbing down with a sterile glass rod, and small quantities measured from a graduated pipette are used for investigation. For estimating the total number of organisms present in the portion of soil used, small quantities, say 0·1 c.c. and 1 c.c., of the fluid are added to melted tubes of ordinary alkaline peptone gelatin; after being shaken, the gelatin is plated, incubated at 22° C., and the colonies are counted as late as the liquefaction, which always occurs round some of them, will allow. From these numbers the total number of organisms (which grow in gelatin) in a given amount of soil can be calculated.

In certain cases it may be necessary to investigate the anaerobic organisms of the soil, *e.g.* B. tetani. The inquiry is necessarily of a qualitative character and the methods to be adopted are those described for the isolation of these organisms (*vide* pp. 80, 527). Information may be acquired by the injection of small portions of the soil into animals (guinea-pigs, mice).

The numbers of bacteria in the soil vary very much. According to Houston's results, fewest occur in uncultivated sandy soils, these containing on an average 100,000 per gram. Peaty soils, though rich in organic matter, also give low results the acidity of such soils inhibiting free bacterial growth. Garden soils yield usually about 1,500,000 bacteria per gram, but the greatest numbers are found in soils which have been polluted by sewage, when the figures may rise to several millions.

Besides the enumeration of the numbers of bacteria present in a soil, an important question in its bacteriological examination is the types of bacteria present in any particular case. Practically this resolves itself into studying the most common bacteria present. Frequent soil organisms are B. mycoides, B. subtilis, and allied types (*vide* p. 396), streptothrix-like organisms, B. proteus, and the nitrifying bacteria; but from the public health standpoint it is obviously of more importance to ascertain the presence of organisms indicative of pollution by sewage, *e.g.* typical B. coli, B. welchii, and streptococci (see p. 739 under Water).

For the detection of these bacteria the following procedures may be recommended :

(*a*) *The B. coli Group* (see Chapter XV.).—A third of a gram of soil is added to 10 c c. of bouillon, shaken up, and loopfuls are spread on one or more plates of MacConkey's lactose neutral-red agar. After twenty-four hours' incubation in an inverted position, any red colonies are picked off and subjected to the tests for typical B. coli, detailed in Chapter XV. If it is necessary to ascertain the actual B. coli content of the soil, varying amounts of the soil emulsion are added to tubes of MacConkey's fluid medium, as in water examination (*q.v.*). The presence of non-lactose fermenters may also claim attention in the examination of such plates, and these would be studied for purposes of identification by the methods described in Chapter XV.

(*b*) *The Bacillus welchii* (see Chapter XXI.).—To search for this organism I gram of the soil is thoroughly distributed in 100 c.c. sterile bouillon, and of this 1 c.c , 0·1 c.c., and 0·01 c.c., are added each to a sterile milk tube. These are heated to 80° C. for ten minutes, and then cultivated anaerobically at 37° C. for forty-eight hours. If the characteristic " stormy clot " reaction is developed, then it may be assumed that this organism is present (*vide* p. 547).

(*c*) *Fæcal Streptococci* (see Chapter VII.).—The best method to employ is that of Prescott and Winslow modified by Mair. This depends on the fact that when B. coli and streptococci are growing together in glucose broth, as the medium becomes acid the streptococci tend to outgrow the B. coli. If lactose neutral-red agar plates be made at this stage, the colonies of streptococci, being small and intensely red, can be distinguished from the larger colonies of the B .coli. They can then be picked off for investi-

gation. It is evident that here the method must be adopted of taking as a measure of the number of streptococci present, the least quantity of the original fluid in which evidence of their presence can be detected.

We may now give in brief the results obtained by the application of such methods. With regard to the typical B. coli, its presence in a soil must be looked on as indicative of recent pollution with excremental matter. The presence of B. welchii is also evidence of such pollution, but from the fact that this is a sporing organism the pollution may not have been recent. With regard to the streptococci, on the other hand, on account of their feeble viability outside the animal body, their presence is to be looked on as evidence of extremely recent excremental pollution.

While such means have been advanced for the obtaining of indirect evidence of pollution of soil, investigations have also been conducted with regard to the viability in the soil of pathogenic bacteria, especially of those likely to be present in excreta, *e.g.* the typhoid and cholera organisms, etc. The solution of this problem is attended with difficulty, as it is not easy to identify these organisms when they are present in such bacterial mixtures as naturally occur in the soil. Further, bacteria when growing together often influence each other's growth in an unfavourable way. For instance, it has been found that the B. typhosus, when sown in an organically polluted soil which has been sterilised, can maintain its vitality for fifteen weeks, but if the conditions occurring naturally be so far imitated by sowing it in soil in the presence of a pure culture of a soil bacterium, it is found that sometimes the typhoid bacillus, sometimes the soil bacterium, in the course of a few weeks, or even in a few days, disappears. Further, the character of the soil exercises an important effect on the results; for instance, the typhoid bacillus soon dies out in a virgin sandy soil, even when it is the only organism present. In experiments made by sowing cultures of V. choleræ and certain other pathogenic organisms in plots in a field, it was found that after forty days, at the longest, they were no longer recognisable. Further, it is unlikely that strictly pathogenic organisms, even if they remain alive, can multiply in soil under natural conditions. In the case of a sporing organism such as the B. anthracis, the capacity for remaining in a quiescent condition of potential pathogenicity is, of course, much greater. The most important principle to be deduced from these experiments is that the ordinary conditions of soil rather tend to be unfavourable to the continued existence of

pathogenic bacteria, so that by natural processes soil tends to purify itself. It must, however, be noted that such an organism as the typhoid bacillus can exist long enough in soil to be a source of danger under certain conditions.

WATER

In the bacteriological examination of water three lines of inquiry may be followed. First, the number of viable bacteria per cubic centimetre may be estimated. Second, the kinds of bacteria present may be investigated. Third, it may be necessary in the case of a particular organism, if present, to ascertain in what number per c.c. it occurs.

Methods.—*Collection of Samples.*—In all water examinations it is preferable that the primary culture media (*i.e.* those to which the water is actually to be added) should be inoculated at the spot at which the sample is collected. When this is not possible, the samples should be packed in sawdust and ice and the primary inoculations made as soon as possible. Otherwise the bacterial content may alter, and an erroneous idea of the number present will be obtained. Immediately after collection a slight diminution in numbers may be observed, but at any rate after six hours an increase over the initial numbers is manifest.

When samples have to be taken for transport to the laboratory, these are best collected in 8-ounce stoppered bottles, which are to be sterilised by dry heat (the stopper must be sterilised separately and not inserted till the bottle has cooled, otherwise it will be so tightly held as to make removal very difficult).

In the case of water taken from a house tap, the water should be allowed to run for some time before the sample is taken, as water standing in pipes in a house is under very favourable conditions for bacterial multiplication.

With river waters it is best to immerse the sampling bottle and then remove the stopper with forceps. Care must be taken not to touch the river bed, as the vegetable matter covering it contains many organisms. When water has to be taken from below the surface of a well or lake, a weighted ~~sample~~ bottle must be used. Several special bottles have been devised for such a purpose. Quite good results are obtained by tying two short lengths of string to the neck and stopper of an ordinary bottle respectively; any required length of string can afterwards be joined to these. A piece of lead is attached to the bottom of the bottle by wires passing round the neck. The whole is then wrapped in paper and sterilised. For use the bottle is carefully lowered to the required depth by the string attached to the neck, the stopper is jerked out by means of the other string, and the bottle filled. If the bottle and stopper be rapidly jerked through the topmost layers, contamination with surface bacteria is not a serious factor.

Counting of Bacteria in Water.—This is done by adding a given quantity of water to 10 c.c. of liquefied agar, plating, and counting

the colonies which develop. The amount of water added depends on its source, and varies from 0·1 c.c. of a water likely to have a high bacterial content to 5 c.c. of a purer water. The plates should be duplicated and one incubated at 37° C. the other at 22° C. The medium should be standardised so as to be +10 according to Eyre's scale (*vide* p. 43), *i.e.* alkaline to litmus. It has been customary to use a gelatin medium for low temperature incubation, but it is found more convenient to employ nutrient agar throughout. The plates incubated at 22° C., give an idea of the numbers of purely saprophytic bacteria present; the plates incubated at 37° C., those which grow at blood heat. As the intestinal bacteria grow at 37° C., the determination of the numbers at this temperature is important. The counts on the two media usually differ according to the prevalence of saprophytic or parasitic organisms respectively. In the case of the plates at 37° C. usually forty-eight hours' incubation is allowed before the colonies are counted, but the plates at the lower temperature should be incubated longer before counting to facilitate recognition of the colonies.

Detection of the Presence of Special Organisms.—(*a*) *The B. coli Group.*—In ordinary public health work, it may be taken that the most frequent and important inquiry with regard to a water is directed to the investigation of the presence or absence of the B. coli and its congeners. Here the method adopted is to determine the smallest quantity of a water which gives evidence of containing organisms of this type. In applying any method with this object in view it is, we consider, absolutely necessary that the primary inoculations should be made at the spot at which samples are collected.

The usual method is to use as the primary culture medium one of the bile-salt preparations, of which the best is MacConkey's bile-salt lactose peptone water to which litmus or neutral-red has been added. In this medium the members of the B. coli group cause changes resulting in the formation of acid and gas. It is thus convenient to put the medium into Durham's fermentation tubes. In practice we employ 2-ounce cylindrical medicine bottles 4½ inches high by 1½ inches in diameter stoppered with cotton wool. The medium, along with the inverted test-tube, is placed in these. It is customary to test for the presence of the organisms in any sample by adding to a series of such tubes the following quantities of the water: 50 c.c. (two samples), 20 c.c., 10 c.c., 5 c.c., 1 c.c., and, it may be, in specially suspicious waters, 0·5 c.c., 0·1 c.c., and even 0·01 c.c. The result is estimated in terms of the smallest amount of water which leads to acid and gas formation. By starting with a concentrated MacConkey's mixture, it is arranged that, when the sample is added, the resulting fluid shall be of the concentration of MacConkey's medium as ordinarily prepared. Thus, the bottle to which the 50 c.c. sample is to be added contains 10 c.c. of a six-fold concentration of MacConkey's medium. For the 20 c.c. sample, 20 c.c. of a medium of double strength; for 10 c.c., 10 c.c. of the double strength medium; and for 5 c.c., 5 c.c. of double strength medium. For smaller samples, we simply use 5 c.c. of the ordinary MacConkey's medium.

For the taking of the samples, sterile 8-ounce stoppered bottles are convenient, and for each sample it is necessary to have sterile 25 c.c., 10 c.c. (graduated to tenths), and 1 c.c. (graduated to

hundredths) pipettes. The armamentarium being thus simple, there is no difficulty in carrying out the necessary manipulations at the spot where the sample is collected.

The tubes are incubated for forty-eight hours, and it is well to read the results at the end of the first twenty-four hours also. The formation of acid and gas in the tube is usually recognised as "presumptive evidence" of the presence of members of the B. coli group, but it is usual to investigate further the bacteria giving rise to this change and determine whether they are "typical" or "atypical" B. coli. With this end in view, each bottle in which acid and gas is present is shaken, two or three loopfuls are placed on a plate of MacConkey's neutral-red bile-salt lactose agar and spread over the surface as in making successive stroke inoculations. The plates are incubated for twenty-four hours. As typical B. coli produces acid in lactose, any colonies of such an organism are of a rose-red colour. These are then picked off, sloped agar tubes are inoculated and used for the further investigation of the properties of the bacterium isolated.

The following tests should then be carried out by the methods described on pp. 399, 400: gelatin liquefaction, indol formation, fermentation of lactose, dulcite, saccharose, adonite, inosite.

It is well in dealing with the neutral-red lactose agar plates to inoculate a lactose peptone water tube from all the kinds of colonies present, whether these are red or not, as sometimes an organism which is really a lactose fermenter does not produce a red colour on the solid medium.

The object of growing suspicious colonies on a range of media such as that given, is to enable typical B. coli to be recognised when present. This subject has been more fully discussed in Chapter XV. Here it may be said that for work on water two standards have been used in this country: first, that of Houston, who recognises as typical qualities the following:—fluorescence in neutral-red broth, production of acid and gas in lactose peptone water, production of indol, production of acid and clot in litmus milk—so-called "flaginac" reaction; secondly, that of the English Committee of 1904, which, on the one hand, laid stress on the additional feature of non-liquefaction of gelatin, and on the other, attached less importance to the occurrence of fluorescence. The characters of a typical B coli on which, in our opinion, special stress should be laid are: absence of gelatin liquefaction, fermentation (with acid and gas production) of lactose, absence of fermentation of inosite, and production of indol from peptone water. The significance of the *Voges-Proskauer* and the *methyl-red reactions* is referred to on p. 400.

(*b*) *B. welchii and streptococci.*—The methods for the detection of these organisms are those which have already been given (p. 735).

The detection of *B. welchii* has been extensively used in water examination as a means of proving the presence of excretal pollution. The milk culture test described on p. 547 has long been spoken of in the literature as the "*B. enteritidis sporogenes*" *test.* The organism responsible for the "stormy clot" reaction is now known to be *B. welchii.*

Much work has been devoted to the question of the fæcal streptococci presenting specific characters by which they could be differentiated from other streptococci. It has been found that the pre-

vailing type of organism is one which produces acid and clot in milk, reduces neutral-red, and ferments saccharose, lactose, mannite, and salicin. It corresponds to the *Streptococcus fæcalis*. The important point in this connection is to recognise that streptococci of such a type exist in great numbers in human fæces, and that when in any circumstances fæcal contamination is suspected, the isolation of streptococci strengthens the suspicion.

With regard to the objects for which the bacteriological examination of water may be undertaken, though these may be of a purely scientific character, they usually aim at contributing to the settlement of questions relating to the potability of waters, to their use in commerce, and to the efficiency of processes undertaken for the purification of waters which have undergone pollution. The last of these objects is often closely associated with the first two, as the question so often arises whether a purification process is sufficiently effective to make the water again fit for use.

Water derived from any natural source contains bacteria, though, as in the case of some artesian wells and some springs, the numbers may be very small, *e.g.* 4 to 100 per c.c. In rain, snow, and ice there are often great numbers, those in the first two being derived from the air. Great attention has been paid to the bacterial content of wells and rivers. With regard to the former, precautions are necessary in arriving at a judgment. If the water in a well has been standing for some time, multiplication of bacteria may give a high numerical count. To meet this difficulty the well ought, if practicable, to be pumped dry and then allowed to fill, in order to ascertain the bacterial content of the water entering the well. Again, if the sediment of the well has been stirred up, a high bacterial count is obtained. Ordinary wells of medium depth contain from 100 to 2000 per c.c. With regard to rivers, very varied results are obtained. Moorland streams are often fairly pure. In an ordinary river the numbers present vary at different seasons of the year, whilst the prevailing temperature, the presence or absence of decaying vegetation, or of washings from land, and dilution with large quantities of pure spring water, are other important factors. Thus the Franklands found the rivers Thames and Lea purest in summer, and this they attributed to the fact that in this season there is most spring water entering, and very little water as washings off land. In the case of other rivers bacteria have been found to be fewest in winter. A great many circumstances must therefore be taken into account in dealing with mere enumerations of water bacteria, and such enumerations

are only useful when they are taken simultaneously over a stretch of river, with special reference to the sources of the water. Thus it is usually found that immediately below a sewage effluent the bacterial content rises, though in a comparatively short distance the numbers may markedly decrease, and it may be that the river as far as numbers are concerned may appear to return to its previous bacterial content. The numbers of bacteria present in rivers flowing through inhabited districts therefore vary greatly.

The bacterial count is of great use in estimating the efficacy of the filter-beds of a town water-supply. These usually remove from 95 to 98 per cent. of the bacteria present, and a town supply as it issues from the filter-beds should not contain more than 100 bacteria per c.c. Again, it is found that the storage of water effects a very marked bacterial purification. Thus Houston has shown in one series of observations that while 93 per cent. of samples of raw river Lea water contained B. coli. in 1 c.c. or less, in the stored water 62 per cent. of the samples showed no B. coli to be present in 100 c.c. According to Coplans, however, the diminution is not necessarily due to the organisms being killed; the real cause may be the agglutination of the bacteria following on changes in the electric conductivity which take place in stored water.

Much more important than the mere enumeration of the bacteria present in a water is the question whether these include forms pathogenic to man. The most important of these are the typhoid-paratyphoid group, B. dysenteriæ, and, in certain circumstances, the cholera vibrio. On account of the small numbers which may be present in a dangerous water, the direct isolation of these organisms is usually impracticable and in any case unnecessary for the condemnation of an unsafe water supply. There is no doubt that the typhoid and cholera bacteria can exist for some time in water—at least this has been found to be the case when sterile water has been experimentally inoculated with these bacteria. But to what extent the same is true when they are placed in natural conditions, which involve their living in the presence of other organisms, is unknown. For public health purposes we therefore seek for the presence of indirect bacteriological evidence which might point to the contamination of a water by human excreta. If this be found we deduce that the water is dangerous, as organisms from any case of intestinal disease occurring in the catchment area may find access to it. The criterion here adopted is the determination of the numbers of typical B. coli present in the water. Klein

and Houston have pointed out that, in crude sewage, members of the coli group are practically never fewer than 100,000 per c.c. In these circumstances the presence of typical B. coli in a water is the best indirect evidence of the possibility of disease organisms of intestinal origin being likely to gain access to that water. It must, however, be at once clearly recognised that these organisms are only as it were indicators, and so far as the potability of any water is concerned, evidence is wanting that they are under ordinary circumstances actually harmful to man.

The difficulty, however, is that (except in the case of water from artesian wells) if a sufficient quantity be taken, evidence of the presence of B. coli will always be found. This arises from the fact that this organism is present also in the excreta of birds and other animals, and it is impossible in the present state of knowledge to distinguish between organisms coming from these different sources. Thus even in the moorland waters so much used for urban supplies, there may be a fairly high content of B. coli—without the least evidence of these being derived from human sources, and the consumption of such a water, even in an unfiltered condition, may be perfectly safe. There is thus the greatest difficulty in the interpretation of bacteriological results in the case of raw waters, and it is impossible to set up any absolute standards of the bacteriological purity of a water based only on the estimation of the numbers of B. coli present. In any particular case the results must be considered along with those of chemical analysis and inspection of the locality. The difficulty is greatest when dealing with water derived from sewage-contaminated rivers, from agricultural land, and from surface wells. With regard to the first two sources, the water should never be used in an unfiltered condition, and with regard to the last, every case must be considered on its own merits. In addition to filtration, chlorination of such waters has come to be a usual practice. It may be said that under ordinary circumstances an inspection of the surroundings and an unfavourable chemical analysis are sufficient to condemn a water, even if a bacteriological examination showed the absence of B. coli in large samples; and further, if in a suspicious locality the bacteriological analysis yielded a bad result, the water ought to be condemned even when from the chemical analysis it could be passed. If any general standard is to be applied it is probably safe to say that when typical B. coli is found in 10 c.c. or less of a water from a suspicious locality it is unsafe for human consumption.

The examination for the presence of B. coli finds a further

application in determining the efficiency of a filtration process, and here it is extraordinarily delicate. While again it is difficult to lay down an absolute standard of purity, the filtration methods in use are, if properly worked, capable of delivering an effluent which does not yield B. coli in amounts less than 100 c.c., and such a degree of efficiency should in all cases be aimed at.

As the B. coli is fairly widespread in nature, Klein and Houston have held that valuable supporting evidence is found in the presence of the " B. enteritidis sporogenes " (B. welchii) and of streptococci, both of which are constant inhabitants of the human intestine. The spores of the former are numerous in sewage, and the presence of the latter can always be recognised in 0·001 grm. of human fæces. The significance attached to the presence or absence of these organisms has already been referred to on p. 736.

It should be emphasised that in water artificially polluted with sewage containing intestinal bacteria, these can be detected by bacteriological methods in mixtures from ten to a hundred times more dilute than those in which the pollution can be detected by purely chemical methods.

The Isolation of the Typhoid-Paratyphoid Group from Water.—Though the typhoid bacillus has been isolated, on occasions, from polluted waters responsible for outbreaks of enteric fever, the technical difficulty of its isolation from a water in which it is likely to be present, at the most only in very small numbers and also along with a greater number of other organisms, renders the procedure hardly practicable as a routine method. In attempting to isolate this organism, it is necessary to utilise some method of concentrating or enriching it in the specimen, and a relatively large quantity of water must be tested for the purpose. Various methods have been recommended. Among these, reference may be made to the following :

(1) *Concentration by alum precipitation* (*Willson's method*).—To a litre of the water are added 5 c.c. of a 10 per cent. solution of alum. A precipitate of aluminium hydrate forms, and after the water has been thoroughly shaken, is allowed to settle out and finally separated by centrifuging. Plates of MacConkey's medium are then inoculated with the deposit.

(2) *Enrichment by brilliant green* (*Browning, Gilmour and Mackie*).—The general principle of this method is referred to on p. 59. To 900 c.c. of the water in a stoppered sterile flask is added 100 c.c. of a 10 per cent. sterile solution of peptone, 5 grams of sodium chloride, and 5 c.c. of a 1 : 1000 solution of brilliant green in distilled water. In this way the water is converted into a suitable culture medium and the brilliant green, in virtue of its selective action, tends to enrich organisms of the typhoid-paratyphoid group while suppressing B. coli. After incubation for twenty-four hours, sub-

inoculations are made on MacConkey plates and suspicious colonies investigated by the procedure detailed on p. 438. Subinoculations may also be made after forty-eight hours' incubation.

(3) *Caffeine method.*—Caffeine has also been used for enriching B. typhosus and inhibiting B. coli in cultures from water. To 900 c.c. of the suspected water are added 10 grams nutrose dissolved in 80 c.c. of sterile water, and 5 grams of caffeine dissolved in sterile distilled water, heated to 80° C. and cooled to 55° C. before addition. After mixing the ingredients, there is added 10 c.c. of 0·1 per cent. crystal violet. The flask is incubated at 37° C. for twelve hours, and then plates of MacConkey's medium are inoculated from it.

The Isolation of Vibrio Choleræ from Water.—For this purpose, advantage is taken of the enrichment of this organism when growing in an alkaline peptone solution: 900 c.c. of the suspected water, to which have been added 100 c.c. of a sterile 10 per cent. solution of peptone (rendered neutral to phenol-phthalein, *i.e. alkaline* to *litmus*) and 5 grms. of sodium chloride, are distributed in stoppered sterile Erlenmeyer flasks so that each flask contains only a shallow layer of the mixture. The flasks are incubated at 37° C., and if vibrios grow they may be isolated and identified by the methods described on p. 459.

Sewage

It is sometimes necessary to examine the bacterial content of sewage, especially in connection with the efficiency of purification works. The main lines of inquiry are here the same as for water, and the general methods are identical, the only modification necessary being that, in consequence of the high bacterial content, much smaller quantities of the raw material must be worked with, *e.g.* a series of decimal dilutions of the sewage. With regard to the numbers of bacteria in sewage, these may vary from a million to ten millions or even more per c.c., and here of course the question of the presence of intestinal organisms of the coli group is of great importance. The numbers of these are large, and members of the group may be detected in 0·000001 c.c. or less. The numbers present are frequently considerably reduced by purification methods, but it is to be noted that, even when such methods are most successful, B. coli may yet be present in considerable quantities. This is especially true in Britain, where sewage is much more concentrated than it apparently is in America. In the latter country, purification may yield effluents in which B. coli can be detected in only 0·001 c.c. By no purification method has the production of a potable water been attained, and the high content of effluents in B. coli suggests that the passage of typhoid bacilli through a purification system is possible.

The part which bacteria play in the purification of sewage

constitutes a question of great interest, to which much attention has been directed. The methods adopted for the biological purification of sewage may be divided into two groups. In the first of these, the sewage coming from the mains is run on to a bed of gravel, clinker, or coke, on which it is allowed to stand for some hours. The effluent is then run out through the bottom of the bed, which is allowed to rest for some hours before being re-charged. In a modification of this method the sewage is allowed to percolate slowly through a bed consisting of large porous objects, such as broken bricks or large pieces of coke, and here the percolation may be constant, no interval of rest being given. The bacterial processes which take place in these two methods are, however, probably closely similar. In the second, the essential feature is a preliminary treatment of the sewage in more or less closed tanks ("septic tanks"), where the conditions are supposed to be largely anaerobic. This method was originally adopted at Exeter, Sutton, and Yeovil in this country, and in America by the State Board of Health of Massachusetts. In the explanation given of the rationale of this process, sewage is looked on as existing in three stages. (1) First of all, *fresh sewage*—the newly mixed and very varied material as it enters the main sewers. (2) Secondly, *stale sewage*—the ordinary contents of the main sewers. Here there is abundant oxygen, and as the sewage flows along, there occurs by bacterial action a certain formation of carbon dioxide and ammonia, which combine to form ammonium carbonate. This is the sewage as it reaches the purification works. Here a preliminary mechanical screening may be adopted, after which it is run into an airtight tank—the septic tank. (3) It remains there for from twenty-four to thirty-six hours, and becomes a foul-smelling fluid—the *septic sewage*. The chemical changes which take place in the septic tank are of a most complex nature. The sewage entering it contains little free oxygen, and therefore the bacteria in the tank are probably largely anaerobic, and the changes which they originate consist of the formation of comparatively simple compounds of hydrogen with carbon, sulphur, and phosphorus. As a result, there is a great reduction in the amount of organic nitrogen, of albuminoid ammonia, and of carbonaceous matter. The last is important, as the clogging of ordinary filter-beds is largely due to the accumulation of such material, and of matters generally consisting of cellulose. One further important effect is that the size of the particles of the deposited matter is decreased, and therefore it is more easily broken up in the next stage of the process. This consists in

running the effluent from the septic tank on to filter-beds, preferably of coke, where a further purification process takes place. By this method there is first an anaerobic treatment, succeeded by an aerobic; in the latter the process of nitrification occurs by means of the special bacteria concerned. The results are of a satisfactory nature, there being often a marked diminution in the number of coliform organisms present.

In the earlier stages of any sewage purification, there is little doubt that the albuminous material present is being split up by ordinary putrefactive bacteria. In the mains and where open systems of purification are at work, aerobic forms play the chief part, while in the closed methods anaerobic organisms are those chiefly concerned. In contact and percolating systems there is evidence that at first the purifying action of bacteria is materially furthered by physical processes. Thus Dunbar has shown that when such a substance as coke is used in a sewage filter-bed a considerable amount of the albuminous material is removed in a very few minutes by adsorption; for albumin, being of a colloidal nature, is readily deposited under such circumstances in the pores of the coke in the form of films. After a time such a filter-bed becomes clogged, but on access of oxygen being allowed, it regains its adsorptive properties—probably from the oxidation of the material adsorbed.

During this stage, as in the whole purification process, four, and it may be five, processes are at work: First, the action of ordinary bacteria splitting up the higher albuminous molecules; secondly, the action of nitrifying bacteria building up nitrites and nitrates from ammoniacal products; thirdly, the action of denitrifying bacteria which reduce nitrates to lower gaseous oxides and to free nitrogen (the presence of which in filter-beds can be demonstrated); fourthly, the action of higher forms of vegetable and animal life; fifthly, it is possible that direct chemical oxidation of the earlier products of bacterial action may occur, and in any case the access of an abundant oxygen supply to adsorbed material hastens its destruction. It is possible, as is indicated by the work of Lorrain Smith and of Mair, that perhaps too little weight has been attached to the parts played by the two last processes specified, for in the later stages of the purification process there is a very marked diminution in the number of bacteria present in the filter. Much further work, however, is necessary before the part to be assigned to each factor in operation can be properly estimated.

Further, the details of the essentially bacterial part of the process are obscure, and the relative parts played, even in an open purification process, by aerobes on the one hand, and anaerobes on the other, are little understood. When sewage is drained off to rest a filter-bed, great quantities of oxygen are sucked in, but as to how long the bed thus remains aerated, authorities differ—some maintaining that oxidation processes persist even after the bed has been re-charged, while others state that soon the oxygen in the resting bed is consumed, and its place taken by carbon dioxide and nitrogen. Certainly, at certain stages of the purification process large amounts of free nitrogen come off the bed, but whether at such periods

anaerobic bacteria are or are not in the ascendant is not known. It is probable that, from the practical standpoint, the later stages of purification should take place with free oxidation, as when anaerobic bacteria are active at this point a very offensive effluent is produced.

The effluent from a sewage purification system may contain as many bacteria as the sewage entering, but there is often a marked diminution. It is said by some that pathogenic bacteria do not live in sewage. The typhoid bacillus has been found to die out when placed in sewage, but it certainly can live in this fluid for a much longer period than that embraced by any purification method. Thus the constant presence of B. coli and other intestinal organisms which has been observed in sewage effluents must here still be looked on as significant, and it is only by great dilution and prolonged exposure to the conditions present in running water that such an effluent can become suitable for forming a part of a potable water.

Milk

The bacteriology of milk presents two aspects, the economic and the hygienic, with the latter of which this chapter is mainly concerned. It may be said that the bacteriological condition of milk, as sold in the large communities of this country, is far from satisfactory from the hygienic standpoint, and the hygienic aspects of milk bacteriology are therefore of paramount importance in preventive medicine. In the bacteriological sense, milk is perhaps the most impure of foods, and therefore a great potential source of infection. Primarily, cow's milk is a sterile fluid, but from the time it flows along the large ducts of the udder, which may contain bacteria of various types, it becomes progressively contaminated. It is an excellent culture medium, especially for intestinal organisms, and any contamination is therefore progressive unless at very low temperatures. The main sources of the bacteria always found in fresh milk are the external surface of the udder, the hands of the milkers, utensils in which the milk is collected, and often dust that has gained access to the milk; but under unhygienic conditions of collection and distribution large numbers may be added by gross pollution. Under the most favourable conditions, fresh milk contains about five hundred organisms per c.c., but the content may rise to thousands under unhygienic conditions, and it has been shown that the numbers of bacteria may be easily controlled by attention to the cleanliness of the cowhouse, by grooming of the animals, washing the udder before milking, etc. There is some

evidence that for a short time after milk is withdrawn a slight diminution of the bacterial content may take place, but before it reaches the consumer, especially in city supplies and in warm weather, the bacterial content of apparently fresh milk may rise to several hundred thousands, or even millions per c.c. The organisms present are mainly intestinal bacteria, *e.g.* the B. coli group, lactic acid bacilli, sporing aerobes and anaerobes, and streptococci, and this is specially significant of the nature of the contamination to which milk is subjected in the process of collection.

The Souring of Milk.—Under ordinary conditions the first evidence of bacterial activity, and from the economic standpoint the most important, is the occurrence of souring due to the formation of lactic and other allied acids, and the action of these on the albuminous constituents is one of the factors in curdling. The subsequent changes vary with the bacteria present, but ultimately these lead up to putrefaction of the ordinary type. The importance of the souring of milk has caused much attention to be devoted to the process, and a variety of bacteria have been described by various observers.

Three main types occur. (*a*) The first of these is the *Streptococcus lacticus*, originally described by Kruse. This is an oval coccus often lanceolate in shape and resembling the pneumococcus, Gram-positive, and showing little tendency to chain formation. Its optimum temperature is from 30° to 35° C. On agar plates the colonies are small and similar to those of other streptococci. In gelatin stabs there is rather a scanty development and no liquefaction. In milk there is considerable variation in the amount of lactic acid produced, and the curd is soft and uniform. Glucose, lactose, saccharose, and mannite are fermented. *There is no gas production.* The organism is regarded as non-pathogenic.

(*b*) *The lactic acid bacilli* (*lactobacilli Beijerinck*) are a group of organisms certain of which occur normally in the intestine of various mammals and are specially numerous in the young animals before weaning. They are non-sporing, non-motile, Gram-positive organisms, non-liquefiers of gelatin; they show great pleomorphism, and the same strain may present marked differences in microscopic appearance according to the conditions under which it is growing. They are highly resistant to acid, and some will grow in the presence of 1 per cent. acetic acid ; on this account they are called *acidophile* or *aciduric*. Some are thermophilic and flourish at 50° C. Among these organisms B. acidophilus and B. bifidus may be specially mentioned as occurring in the human subject. No pathogenic properties have been attributed to these bacteria ; but a related organism, *B. acidophilus odontolyticus*, has been described by M'Intosh and his co-workers as the cause of dental caries. The Boas-Oppler bacillus is found in the stomach contents in states in which HCl is absent, *e.g.* gastric cancer, and lactic fermentation is occurring.

B. acidophilus is a rather long, stout bacillus (1 μ or more broad), with a tendency to curl at the ends ; but a more slender form which tends to be in chains also occurs (Fig. 208). B. acidophilus may be recovered from fæces by plating in 1 per cent. peptone agar containing 2–4 per cent. lactose, or, better, in whey agar [1] or after preliminary enrichment in neutral broth to which 1 per cent. acetic acid has been added, and incubation at 37° C. The colonies are very minute, and for their detection one must examine the culture under the low power of the microscope ; they are of two types, the one a delicate feathery growth (Fig. 209), the other rounded or fusiform and often with fringe-like projections (" crab colonies "). *B. bulgaricus* is a related organism ; it has the same microscopic characters and forms similar feathery colonies ; it can be differentiated from B. acidophilus by the fact that the latter forms acid in 1 per cent. maltose broth in forty-eight hours at 37° C., whereas B. bulgaricus fails to do so ; the latter also does not flourish in the human intestine. Both these organisms are aerobes.

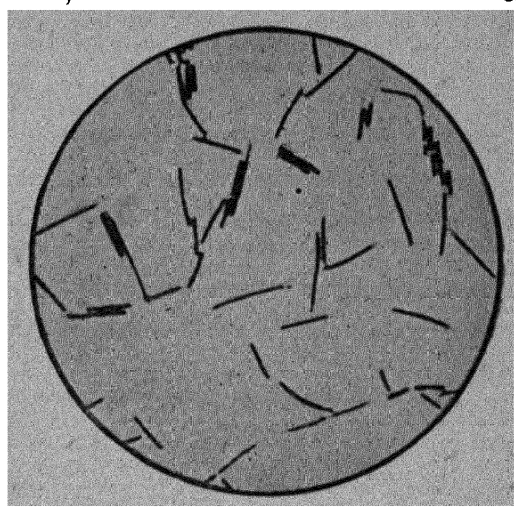


FIG. 208.—*B. acidophilus* from twenty-four hours' growth on agar. Gram's method. ×1000.

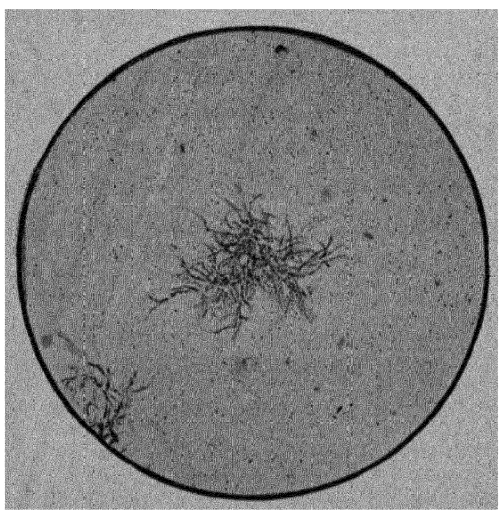


FIG. 209.—Surface colony of *B. acidophilus* on agar plate ; twenty-four hours' growth. ×100.

B. bifidus is the predominant organism in the intestine of young breast-fed infants and in a film of the fæces it appears in practically pure culture (Fig. 210), being 4–5 μ long by 0·7 μ broad, and straight or slightly curved, often with bulbous ends ; but in cultures pleomorphism is marked (Fig. 211). Often the bacilli are only weakly Gram-positive. In order to obtain this organism in culture special conditions are required. Deep tubes of 1 per cent. lactose or glucose

[1] Whey broth is prepared by adding to skimmed milk at 80°–90° C. just sufficient 10 per cent. HCl to precipitate the casein. The fluid is filtered through cotton wool and then through paper and the P_H adjusted to 6·8–7·0 with NaOH. Finally, 0·5 per cent. peptone is added and the mixture autoclaved at 120° C. for fifteen minutes and filtered ; whey agar consists of the above medium plus 1·2 per cent. agar.

broth neutral to litmus to which a small piece of sterile rabbit's kidney has been added, are heavily inoculated with a suspension of the fæces and sealed with a layer of sterile vaselin and are kept at 37° C. for six to eight days. Any gas which forms at first is expelled by remelting the vaselin. Stroke cultures are then made on plates of 1 per cent. glucose agar or Löffler's serum and are incubated anaerobically. After forty-eight hours B. bifidus has formed greyish pin-head colonies resembling those of diphtheroids, which in the next day or two enlarge to 3–4 mm. in diameter (the only other organisms likely to be present are enterococci which form larger, whitish colonies). Rich subcultures on glucose agar and in glucose broth will now be found to grow aerobically.

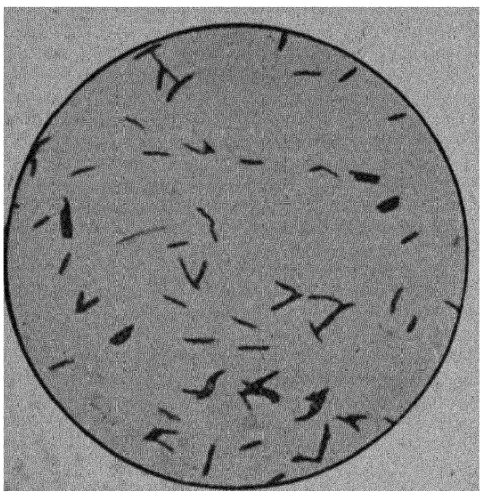


FIG. 210.—*Bacillus bifidus*, as seen in film preparation from fæces of an infant. Gram's method. ×1000.

The above-mentioned organisms do not form indol and fail to produce gas from carbohydrates. A number of similar organisms have been isolated from fermenting milk or carbohydrate materals, some of which are gas producers.

(*c*) The third great group of milk-souring organisms is the *B. coli group*, certain types of which were originally described in connection with this process, *e.g.* B. acidi lactici (*vide* p. 442). These organisms produce both acid and gas from lactose, and curdling of milk by their growth may be associated with gas formation.

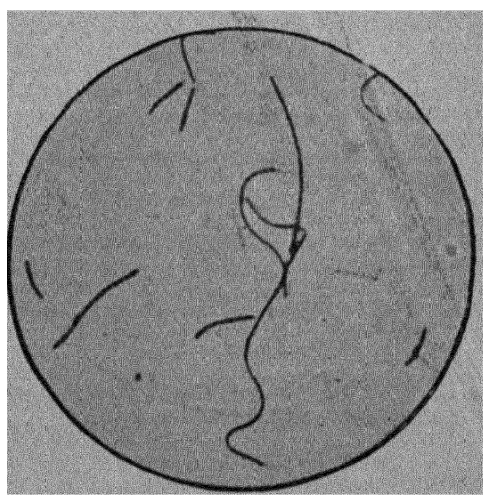


FIG. 211.—*Bacillus bifidus*, from a three-days' culture on agar. Gram's method. ×1000.

As already stated, there occur in milk a number of bacteria of very different morphological and cultural characters with the common capacity of producing lactic and other acids, and the special qualities of any souring process probably depend on the particular combination of bacteria present. There is considerable evidence that the occurrence of souring holds in abeyance for a time the activity of putrefactive organisms whose special characteristic is

the disintegration of the protein molecules. Many changes, which may be denominated economic diseases of milk, are also due to bacteria, *e.g.* the occurrence of ropy milk, bitter milk, and coloured milk.

Pathogenic Organisms in Milk.—From the hygienic standpoint the most important consideration is that of the conditions under which organisms pathogenic to man gain access to milk. These may originate in diseased conditions occurring in the cow, or the milk may become contaminated from cases of human disease. With regard to the former, the two most important are inflammatory and suppurative disease of the udder, and tuberculosis. Thus, mastitis, sometimes with abscess formation, is not infrequent and is due to streptococci of the pyogenes type. The milk in such a case may contain pus and blood-stained serum along with large numbers of streptococci. There is evidence to show that even such milk, and at any rate milk from less acute conditions, finds its way into large milk supplies, and definite outbreaks of streptococcal sore throat and abscess in the cervical glands have actually been traced to such sources. Probably many similar cases of a sporadic kind have a like origin. The B. melitensis may occur in the milk of infected goats, and undulant fever is usually traceable to this source (see Chapter XX.). B. abortus, which is closely related to this organism, may be present in the milk of infected cows, and the question of its pathogenicity to the human subject has been raised (*vide* p. 522). Human cases of foot-and-mouth disease have also been recorded and traced to milk from infected animals. In outbreaks of bacterial food poisoning due to the Salmonella group, cow's milk has been found to be infected with this type of organism and has been responsible for the condition (*vide* p. 428).

Tuberculosis in the cow is a serious source of human tuberculosis arising from the consumption of milk. It should be noted that the disease is exceedingly prevalent in milk cows in this country. The relation of the bovine type of the tubercle bacillus to the human is discussed in Chapter X. Here it need only be said that where tubercular disease occurs in the cow's udder, tubercle bacilli will be found in the milk, and, further, that where generalised tuberculosis occurs in the animal, tubercle bacilli have been found in the milk without evidence of the udder being diseased. The importance of this observation is evident from the fact that a cow with enormous deposits of tubercle in the lungs and peritoneum may to external inspection appear in normal condition. Further, under conditions of unhygienic milking, tubercle bacilli may gain access to milk from the excreta

of infected animals and by contamination of the milk from the air, dust, and filth of a cow-house occupied by tuberculous cattle. Though udder tuberculosis occurs in only 1–2 per cent. of cows or even less, and though only a very small proportion of milk samples from individual cows contain tubercle bacilli, the mixing of milk for market purposes increases the percentage of specimens of tuberculous milk as supplied to the community. This percentage has been found to be alarmingly high, *e.g.* 2–12 per cent. in the English cities according to Buckley in 1921.

Apart from disease conditions of the cow itself, milk may be a disseminating agent from being infected through handling by those suffering from disease. Two diseases occasionally spread in this way are diphtheria and typhoid fever. In the former case the bacilli have been actually isolated from the milk. In the case of typhoid fever the chief danger lies in the milk being contaminated by a " carrier " (see Chapter XV.). In the same way milk may be a source of choleraic and dysenteric infection in countries in which these diseases are prevalent. There is good reason to believe also that cases and outbreaks of scarlatina may owe their origin to milk infected with the specific virus from a person handling the milk while harbouring this organism, *e.g.* a convalescent or an unrecognised case. Similarly a contaminated water used for " watering " milk has been known to give rise to milk-borne outbreaks of enteric fever. House-flies infected with organisms of the typhoid, paratyphoid, and dysentery groups (*vide* p. 418) may contaminate milk with these organisms, and the same applies to the cholera vibrio in countries in which cholera is epidemic.

A very important question in the bacteriology of milk is the milk factor in the causation of infant diarrhœa. It has been long recognised that cow's milk is responsible for a considerable morbidity and mortality among infants from acute diarrhœa, especially during the summer months when, as might be expected, the bacterial content of milk is generally highest; and without considering any specific bacterium as the causal agent, it might be supposed that the ordinary flora of contaminated milk in excessive numbers or the chemical products resulting from their growth, are responsible for such diarrhœal conditions in infants. Specific organisms have, however, been noted in cases and outbreaks of infant diarrhœa, *e.g.* B. dysenteriæ, B. Morgan No. 1 (*vide* p. 435), and milk cannot always be incriminated. Further, apart from actual disease in the cow or in those handling the milk, organisms capable of causing disease in man may gain access from external sources.

Bacteriological Standards and Graded Milks.—Bacteriological standards have now been extensively applied in controlling milk supplies. It may be said that with proper precautions as regards the hygienic collection and distribution of milk, the bacterial content should not exceed 30,000 viable organisms per c.c. The actual factors concerned in reducing bacterial contamination to a minimum need not be detailed here. For practical information on this subject the reader is referred to works on hygiene

In recent years, with a view to ensuring that supplies of cow's milk as free as possible from danger to health should be available to the public, grades of milk have been recognised and sold as such, while standards have been laid down for controlling these graded milks. Thus, in the case of the so-called *certified milk*, the bacterial content should not exceed 30,000 per c c. and B. coli should be absent from 0·1 c.c. ; this milk must be from tuberculin-tested cows and no heat must have been applied to it The *Grade A milk* must not contain more than 200,000 organisms per c c. and B. coli must be absent from 0·01 c.c *Pasteurised milk* is referred to below.

The Sterilisation and Pasteurisation of Milk.—The danger arising from milk being contaminated by disease organisms has caused much attention to be paid to the subject of their destruction before the milk is consumed. The only practical method is sterilisation by heat, and it is fortunate that practically all the important organisms to be considered are non-sporing forms and thus are easily destroyed. To obviate the development of the rather unpleasant taste caused by boiling milk, Pasteur's method of heating the milk for twenty minutes to between 60° and 80° C. has been extensively used. This usually kills all but about 5 per cent. of the organisms present, and will dispose of the hæmolytic streptococci, the tubercle bacillus, and B. diphtheriæ. Sporing putrefactive forms, however, often survive, and unless the pasteurised milk be rapidly cooled, the action of the process as an economic preservative is largely nullified, more especially as the protective milk-souring forms are destroyed. The boiling of milk for two or three minutes will kill all harmful organisms, and although some spores may survive, this is by far the most simple and practical method of sterilisation for domestic application. Boiling has been objected to, however, on account of the destruction of vitamins, *e.g.* the anti-scorbutic principle, and if very young children on an exclusively milk diet be given boiled milk alone, in a certain number of cases scurvy results.

Pasteurisation at a low temperature has now been generally accepted for the disinfection of milk, and this process has been adopted for commercial purposes and recognised by the Health

Authorities under certain conditions, *e.g.* the " Holder " method in which the milk is maintained at a temperature of 62·8°–65·5° C. (145°–150° F.) for thirty minutes, and then immediately cooled. The bacteriological standard laid down is that the pasteurised milk should not contain more than 100,000 organisms per c.c., and that B. coli should be absent from 0·1 c.c. It is doubtful whether pasteurisation on a large scale for commercial purposes is uniformly effective, and this is borne out by the bacteriological examination of specimens of pasteurised milk sold as such. The B. coli content of milk appears to be a valuable and sensitive index of the efficiency of pasteurisation, and effectively pasteurised milk should not contain any coliform bacilli in 1 c.c. The question has also been raised as to whether, in the process of commercial pasteurisation, there is a sufficient margin of safety as regards the destruction of the tubercle bacillus. The thermal death-point of the tubercle bacillus in milk has been investigated by various workers. H. Jenkins has recently shown that milk containing large numbers of tubercle bacilli (artificially and naturally infected) is rendered non-pathogenic (on inoculation into guinea-pigs) by exposure for thirty minutes at 60° C., and that an infected milk passed through a commercial pasteurising plant is also disinfected in the same way. The officially recognised method of commercial pasteurisation would thus appear to allow sufficient margin of safety as regards the destruction of the tubercle bacillus, provided the pasteurising apparatus is operated with reasonable care

Methods.—As regards the enumeration of the total viable bacteria in milk and the estimation of the B. coli content, the methods used in water examination (*q.v.*) may also be applied. Decimal dilutions, 1 : 10, 1 : 100, 1 : 1000, 1 : 10,000, and 1 : 100,000 are made with sterile water in sterile flasks or bottles. 0·5 c.c of each of these is then plated in standardised nutrient agar and the plates are incubated at 37° C. for two days. The most suitable plate, *i.e.* one not overcrowded with colonies, is selected for the enumeration. In this way the number per c c. can be calculated. The B. coli content can be estimated by adding varying amounts to MacConkey's fluid medium in Durham tubes, as in water examination, *e.g.* 1 c c., 0·1 c.c , 0·01 c.c. of the 1 : 10 dilution, 0·1 c.c. of the 1 : 100 dilution, and so on. Acid with gas production is presumptive of the presence of B. coli.

For the detection of tubercle bacilli, a large quantity of the milk, *e.g.* 100 c.c., is thoroughly centrifuged, and from the sediment guinea-pigs are inoculated subcutaneously. The sediment may be examined microscopically for tubercle bacilli by the usual method (*vide* p. 109), but reliance can hardly be placed on the mere finding of acid-fast bacilli, and the animal test must be employed. The occurrence of tuberculosis in the inoculated animals constitutes conclusive evidence (*vide* p. 323). The lesions should be examined for tubercle bacilli in view of the fact that B. abortus, which may occur in cow's milk, can produce a pathological condition in guinea-pigs simulating tuberculosis (*vide* p. 522).

Other pathogenic organisms that may occur in milk, *e.g.* B. typhosus, etc., can be demonstrated by the methods of cultivation appropriate to the particular organism, the sediment of a centrifuged specimen of the milk being used as the inoculum.

While these are the more important methods applicable to the

bacteriological examination of milk in hygiene work, various other procedures have been employed in milk bacteriology; for these, reference should be made to works on agricultural and dairy bacteriology.

Antiseptics

The death of bacteria is judged of by the fact that, when they are placed on a suitable food medium, no development takes place; but, although not killed, bacteria under certain conditions may be deprived of their capacity to proliferate and to produce changes in organic matter. From the importance of being able to kill or inhibit bacteria, an enormous amount of work has been done in investigating the means of doing so by chemical agencies. A substance having such a capacity is called an antiseptic, and the term is usually applied to substances which act in relatively high dilutions. Most antiseptics are general protoplasm poisons harmful to all forms of life, higher as well as lower, so that their use is limited to the inhibiting or killing of bacteria outside the animal body, but still even this is of high importance. Recently, however, some success has been obtained in finding substances whose antiseptic properties can be exerted locally in infected tissues.

Methods.—These vary very much. In early inquiries the amount of an antiseptic necessary to prevent putrefaction, *e g.* in bouillon, urine, etc., was studied; but as bacteria vary in their powers of resistance, the method was unsatisfactory. It is now usual to estimate the effect of an antiseptic on pure cultures of pathogenic microbes, and in the case of a sporing bacterium, the effect on both the vegetative and spore forms is investigated The organisms most used are the Staphylococcus pyogenes, Streptococcus pyogenes, B. coli, and the typhoid, cholera, diphtheria, and anthrax organisms—the latter being employed for testing the action on spores. A good method is to wash off the growth from a young sloped agar culture, and suspend it in a small amount of distilled water, remove coarse particles by centrifuging or filtering through glass wool, add a measured quantity of this fluid to a given quantity of varying dilutions of the antiseptic dissolved in distilled water, then after the lapse of the period of observation, *e.g.* fifteen or thirty minutes, to remove one or two loopfuls of the mixture and place them in a great excess of culture medium; here it is preferable to use melted agar, which is then plated and incubated. In dealing with strong solutions of chemical agents it is necessary to be sure that the culture fluid is in great excess, so that the small amount of the antiseptic which is transferred with the bacteria may be diluted far beyond the strength at which it may be capable of inhibiting growth of the organisms. Sometimes it is possible at the end of the period of action to change the antiseptic into inert bodies by the addition of some other substance, but there is an objection to this procedure if a precipitate results, since the bacteria may be

carried down with the precipitate and may escape the culture test. To test the effects of antiseptics on spores, Koch soaked silk threads in an emulsion of anthrax spores and dried them. These were then subjected to the action of the antiseptic, well washed in water, and laid on the surface of agar. In using this method to test the efficiency of mercuric chloride it was found necessary to treat the organisms with ammonium sulphide, otherwise the antiseptic effect of traces of the mercuric chloride fixed by the spores went on after they were removed to the culture medium. As a rule the method described, in which the small amount of antiseptic adhering to the bacteria is diluted with an excess of culture fluid, can safely be followed, especially when a series of antiseptics is being compared. Krönig and Paul introduced what is known as the "garnet method" for testing antiseptics. In this, small glass beads of equal size are carefully cleaned, dipped in an emulsion of anthrax spores, and allowed to dry. They are then placed in the antiseptic solution, and from time to time some are removed, washed, and well shaken in a measured quantity of water. This is plated, and the number of anthrax colonies developing is counted. In order to test a slowly acting antiseptic, varying concentrations of the latter are added to a constant volume of medium, *e g.* 0·7 per cent. neutral peptone water or sterile serum (previously heated at 56° C.), in a series of plugged sterile tubes; then the test amount of bacteria, *e.g* a diluted young broth culture, is added to each; the tubes are incubated for twenty-four or forty-eight hours at 37° C. and the presence or absence of growth noted by the appearance of turbidity and by subculturing.

Much attention has been paid to the standardisation of antiseptics, and a watery solution of carbolic acid is commonly taken as the standard with which other antiseptics are compared. The procedure in Rideal and Walker's method is to inoculate a fixed volume of a series of dilutions of the antiseptic to be tested, and at the same time a similar volume of a standard solution of carbolic acid, *e.g.* 1 : 150; subcultures from all the tubes are made at short intervals and incubated. The results show (1) the time required to produce sterility with the concentration of carbolic acid used, and (2) the highest dilution of the other antiseptic which also produces sterility in the same time, say 1 : 3000. The latter substance is then stated to have a "carbolic acid coefficient" of 20. It is to be noted that in comparing antiseptics by the above method the question of the organisms being killed at different rates by various substances is not taken into account.

The Action of Antiseptics.—The action of antiseptics depends upon various factors. Thus the medium in which the bacteria are suspended is important; most of the powerful antiseptics are greatly diminished in their action by the presence in the fluid of proteins or minute particles of organic matter which, according to Chick and Martin, act as absorbing agents, and this is the reason why the action of such antiseptics on bacteria in wounds is limited in degree. The presence of water in the medium also plays an important part; thus carbolic acid when dissolved in oil is a much less powerful antiseptic than when in

watery solution ; and 60 per cent. alcohol kills organisms more rapidly than absolute alcohol. Again, the temperature at which the mixture of antiseptic and organisms is kept has a great influence : at 37° C. the lethal action occurs more rapidly and also takes place in higher dilutions than at lower temperatures. Other factors, such as the hydrogen-ion concentration, the degree of ionisation, and the dispersion of the antiseptic, may have a marked effect upon the lethal action of a given substance on bacteria. The first two of these factors are well illustrated by mercuric chloride, the antiseptic properties of which are due to the mercury ions ; increase in hydrogen-ion concentration brought about by adding dilute HCl increases the action, but the presence of NaCl, which reduces the ionisation of the mercury salt, diminishes the antiseptic power. On the other hand, phenol, whose antiseptic effect is due to the molecule as a whole, is increased in its action by the presence of salt. As regards the influence of dispersion, Chick and Martin found that certain antiseptics of the phenol group acted more powerfully when in the form of a fine emulsion than when in true solution, the former condition apparently favouring absorption of the antiseptic by the bacteria. The killing of bacteria by an antiseptic appears to proceed like a chemical reaction, such as the inversion of sugar by acid ; thus Chick has shown that when the antiseptic is in excess, the number of organisms killed in a given time bears a fixed proportion to the number present at the beginning of the period of observation. Antiseptic properties, of course, depend essentially on chemical constitution. Some antiseptics, such as phenol or mercuric chloride, act rapidly, and concentrations which fail to kill the bacteria within a few hours may have little effect in inhibiting their multiplication subsequently. On the other hand, some basic organic dyes are only slowly lethal ; at first they merely prevent proliferation of the organisms (bacteriostatic action), and twenty-four or forty-eight hours are required for the development of their maximum lethal effect. Another property of certain antiseptics is their selective action ; for instance, with crystal violet the concentration required to kill B. coli is five hundred times greater than that which kills staphylococcus. Both of these organisms, however, are practically equal in their susceptibility to mercuric chloride.

Nearly every substance which is not a food to the animal or vegetable body is more or less harmful to bacterial life. Thus neutral salts, *e.g.* NaCl, when present in high concentration act as preservatives by preventing the growth of bacteria, probably

to a great extent through rendering unavailable the water which is essential for their proliferation. But, as has been stated, the term antiseptic is applied to substances which act in comparatively high dilutions. The most important antiseptics are the salts of the heavy metals, certain acids, especially mineral acids, certain oxidising and reducing agents, volatile oils, and a great variety of organic chemical compounds. In comparing different antiseptics it is important to express their activity in terms of the molecular concentrations of the solutions used. When this is done, certain important facts emerge. Thus the compounds of a metal of high atomic weight are generally more powerful antiseptics than those of one belonging to the same series, but of a lower atomic weight. Again, strong acids, *i.e.* those which are highly dissociated, *e.g.* HCl, are more powerfully antiseptic than the weak acids, *e.g.* boric, the H-ions in this case being responsible for the effect, although other factors may also be involved. With regard to oxidising agents and reducing agents, probably the possession of such properties has been overrated as increasing bactericidal potency. Thus in the case of such reducers as sulphurous acid and formic acid, the effect is apparently chiefly due to the fact that these substances are acids. Formic acid is much more efficient than formate of sodium. In the case of permanganate of potassium, which is usually taken as the type of oxidising agents in this connection, it can be shown that the greater amount of the oxidation which takes place when this agent is brought into contact with bacteria occurs after the organisms are killed. The essential chemical characters which determine the antiseptic properties of substances are, however, still obscure.

The Effects of certain Antiseptics.—Here we can only briefly indicate certain results obtained with the more common members of the group.

Halogens.—Chlorine dissolved in water is a very powerful antiseptic provided that practically no other organic material is present in addition to the bacteria. Hence it has been used on a large scale for the sterilisation of drinking-water supplies (1 to 2 parts of chlorine per million parts of water, thirty minutes being allowed for action). At the end of the action any excess of chlorine can be converted into harmless products by adding sodium thiosulphate. *Hypochlorites* and *chloramine-T* (p-toluene sodium sulphochloramide) are similar in their action. These have been used as wound-antiseptics, the hypochlorites being employed in the form of Eusol (Lorrain Smith) and Dakin-Daufresne solution. A fresh 5 per cent. solution of chloramine-T added in the proportion of 2 volumes to 1 volume of tubercular sputum will sterilise the latter in four hours (Uhlenhuth and Hailer). *Iodine* in the form of Tr. iodi is used for

sterilising the skin surface prior to surgical operations. The action of *iodoform* is obscure, as it shows no antiseptic action *in vitro*.

Mercury compounds.—The perchloride is a strong antiseptic, a 1 : 1000 solution being rapidly fatal to all non-spored organisms, and of all the salts of the heavy metals it has been most used It is, however, a powerful poison for higher forms of life. Certain other salts of mercury, such as the oxycyanide, do not corrode metals as the perchloride does. Recently organic compounds of mercury have been introduced, *e.g.* mercurochrome-220 (sodium salt of dibromoxy mercury fluorescein), which, although powerfully antiseptic, are relatively non-toxic for mammalian tissues and therefore are suited for therapeutic purposes

Phenol (*carbolic acid*), since its introduction for surgical purposes by Lister, has been widely used. As it is a much less powerful antiseptic than mercuric chloride, although more rapid in its action, higher concentrations must be employed—usually a 1 : 20 or 1 : 40 solution. It is an active general protoplasm poison. Chemically analogous substances, the *cresols*, are stronger antiseptics than phenol; a mixture of these with soaps, *e.g.* liquor cresolis saponatus (B.P.), has the advantage of detergent as well as antiseptic action and is used as a 5 per cent. dilution for treating infected articles.

Formaldehyde, used as formalin, a 40 per cent. solution in water, is a powerful antiseptic and general poison. It is employed for treating infected articles, and, on account of its being volatile, is used for room disinfection, *e.g.* by spraying with 2 per cent. formalin. It tends to polymerise into inactive compounds.

Basic organic dye-stuffs.—Certain of these are very powerful bacteriostatic agents, and the extremely low concentrations which at first inhibit the activity of the bacteria, finally cause their death, *e.g.* brilliant green and acriflavine (diamino-acridine methochloride). The latter, which is not diminished in its action by serum, especially on account of its low toxicity for mammalian tissues, is suitable for applying to infected wounds.

From the examples which have been given it will be recognised that the choice of an antiseptic and the precise manner in which it is to be employed depend on the conditions under which the bacteria are to be killed. In conclusion, it may be said that experiments *in vitro* alone cannot give accurate information as to the action of antiseptics within the living body, *e.g.* in a wound.

In addition to chemical substances, certain *physical agencies* are harmful to bacteria. The importance of heat as a sterilising agent has already been dealt with. The action of heat depends greatly upon the presence of water, and organisms which are rapidly killed at 100° C. when in watery medium may when dry withstand prolonged heating at this temperature. This effect of water is no doubt related to its action in leading to the denaturation of proteins by heat. The bactericidal effect of radiations is very marked in the case of certain of the ultra-violet rays, but ordinary light, X-rays, and radium emanations are less active.

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[1] Bacteriological literature has become so extensive in recent years that it is impossible to give even a satisfactory summary. In making additions, our endeavour has been twofold, namely, to give references to authors mentioned in the text, and to quote papers in which summaries and references to recent literature may be found. We have retained most of the old references in view of their historical interest.

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CHAPTER XXIV.—Rickettsia Group

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CHAPTER XXV.—Filter-passing Viruses

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CHAPTER XXVII.—Protozoal Diseases

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INDEX

Abrin, 171, 183, 190.
Abscesses in amœbic dysentery, 672.
  pyæmic, 254.
Absorption method for agglutinins, 126.
Achoria, 716.
Achorion schönleinii, 718.
Acid-fast bacilli other than the tubercle bacillus, 329.
Acidophile bacteria, 748.
Acids as antiseptics, 758.
Aciduric bacteria, 748.
Acne bacillus, 262.
Acquired immunity, 175.
    theories of, 213.
Acriflavine as antiseptic, 759.
Actinobacillus, 369, 375.
Actinomyces, characters of, 364.
  cultivation of, 371.
  maduræ, 379.
Actinomycetales, 18.
Actinomycosis, 364.
  diagnosis of, 376.
  lesions in, 369.
  sources of infection, 370.
Active immunity, 176 *et seq.*
Aedes calopus (Stegomyia fasciata), and yellow fever, 591, 592, 599.
Aerobes, 20.
Aerobic organisms, separation of, 73.
" Aestivo-autumnal " fevers, 656.
African horse sickness, 614.
  relapsing fever, 581.
Agar media, 49.
Agglutinating sera, method of obtaining, 127.
Agglutination, 202.
  absorption method, 440.
  of glanders bacillus, 359.
  in Malta fever, 521.
  methods of, 123.
  nature of, 207.
  in plague, 513.
  of tetanus bacillus, 537.
  in tuberculosis, 339.
  in typhus fever, 605.
Agglutinins, absorption method for, 126.
  characters of, 204.
  group and specific, 427.
Agglutinogen, 203.
Agglutinoid, 204.
Aggressins, 165.
Air, bacteriology of, 731.
  methods of examination of, 731.
Alastrim, 619.
Alcaligenes abortus, 522.
  melitensis, 518.
Alexin, 196, 221.
Allergy, 229.
Amboceptor, 196.
Amœba coli, 663.
Amœbæ, non-pathogenic, 668.
Amœbic dysentery, 663.
    lesions in, 671.
    methods of examination in, 674.
Anaerobes, 20.
  cultures of, 77.
  fusiform, 559.
  in infected wounds, 543.
  proteolytic, 545.
  saccharolytic, 545.
  separation of, 80, 83.
  toxins of, 85.
Anaerobic bacilli, 524.
Anæsthetic leprosy, 346.
Anaphylactin, 226.
Anaphylactogen, 226.
Anaphylatoxin, 228.
Anaphylaxis, 223, 537.
  features of, 224.
  passive, 225.
  theories as to, 227.
Anatoxin, 193.
Andrade's indicator, 64.
Aniline dyes, list of, 102.
Aniline oil, dehydration by, 101.
Aniline-oil water, 105.
Aniline-oil-xylol, 101.
Animals, autopsies on, 144.
  inoculation of, 77, 141.
Anopheles and malaria, 647, 658.
Anthrax, 380.
  diagnosis of, 395.

Anthrax, disposal of animal carcases, 392.
immunity against, 392.
in animals, 386.
in man, 389.
protective inoculation against, 393.
spread of, 391.
Anthrax bacillus, biology of, 384.
capsulation of, 385.
characters of, 380.
cultivation of, 382.
experimental inoculation with, 389.
in soil, 736.
organisms related to, 396.
sporulation of, 384.
Anti-abrin, 190.
Anti-anaphylaxis, 225.
Anti-anthrax sera, 393.
Antibacterial serum, 194.
Antibodies, 184.
chemical nature of, 210.
non-specific formation of, 211.
source and nature of, 208.
specificity of, 185.
varieties of, 186.
Anti-cholera inoculation, 458.
Anti-diphtheritic serum, 187.
Anti-dysenteric serum, 434.
Antiformin, 342.
Antigens, for Wassermann reaction, 137.
heterophile, 200.
nature of, 184.
Anti-meningococcic serum, 294.
Anti-plague serum, 513.
Anti-pneumococcic serum, 280.
Anti-ricin, 190.
Antiseptics, 755.
action of, 756.
methods of testing, 755.
standardisation of, 756.
varieties of, 758.
Antisera, therapeutic use of, 212.
Antistreptococcal serum, 195.
Anti-tetanic serum, 536.
Antitoxic action, nature of, 190.
theories of, 191.
serum, 187.
Antitoxin, development of, 188.
standardisation of, 189.
Anti-tubercular sera, 341.
Antivenin, 190.
Arthrospores, 8.
Ascocarps, 711.
Ascoli's thermo-precipitin reaction in anthrax, 395.
Ascomycetes, 710, 711.
Aspergillosis, 723.
Aspergillus, 710.
Aspergillus fumigatus, 723.
herbariorum, 711.
niger, 711.
Attenuation of virulence, 178.
Autoclave, use of, 37.
Autolysis, 28, 164.
Autopsies on animals, 144.
Avian tuberculosis, 328.
Axoneme, 677.

Babesia (*see* Piroplasma), 705.
bigemina, 705.
bovis, 705.
Bacillary dysenterv, 429.
necrosis, 376.
Bacilli, 2, 14.
acid-fast, 329.
stain for, 109.
fusiform, 559.
Bacillus abortus, 522.
in milk, 751.
acidi lactici, 403, 442.
acidophilus, 748, 749.
odontolyticus, 748.
of acne, 262.
actinomycetum comitans, 374.
aerogenes encapsulatus, 545.
aertrycke, 427.
anthracis (*see* Anthrax bacillus), 380.
in soil, 736.
anthracoides, 396.
avisepticus, 515.
bifidus, 748, 749.
of Boas-Oppler, 748.
botulinus, 540.
bovisepticus, 515.
bronchisepticus, 523.
bulgaricus, 749.
butter (Rabinowitsch), 330.
coli, agglutination of, 401.
anaerogenes type, 442.
characters of, 398.
communior, 403.
experimental inoculation with, 248.
group in soil, 735.
isolation of, 401.
lesions produced by, 250.
in milk, 750.
pathogenic properties of, 405.
reactions of, 399.
typical, 402.
typical and atypical, 739.
varieties of, 402.
cuniculisepticus, 515.
of Danysz, 429.
diphtheriæ (*see* Diphtheria bacillus), 463.
dysenteriæ (*see* Dysentery bacillus), 430.

Bacillus, enteritidis (Gaertner), 426.
sporogenes (*see* B. welchii), 545, 739.
fæcalis alkaligenes, 432, 436.
fallax, 550.
fusiform, 559.
of glanders (*see* Glanders bacillus), 353.
IX of von Hibler, 556.
histolyticus, 557.
of Hofmann, 482.
icteroides, 591.
of influenza (*see* Influenza bacillus), 487.
Koch-Weeks, 259.
lacunatus, 260.
lactis aerogenes, 442.
of leprosy (*see* Leprosy bacillus), 346.
of malignant œdema, 551.
mallei, 353.
of melioidosis, 361.
melitensis, 518.
in milk, 751.
" mist " (Moeller), 330.
Morax-Axenfeld, 260.
of Morgan, No. 1, 432, 435.
mycoides, 396.
œdematiens, 555.
œdematis maligni, 551.
paracolon, types of, 405, 432.
paramelitensis, 521.
paratyphosus, A and B, 422.
perfringens, 545.
pertussis, 497.
pestis (*see* Plague bacillus), 502.
phlegmonis emphysematosæ, 545.
proteus, 245, 432, 605.
occurrence of, 252.
pseudo-diphtheria, 482.
psittacosis, 429.
pyocyaneus, 246.
lesions caused by, 251.
of quarter-evil, 557.
of Rauschbrand, 557.
of rhinoscleroma, 362.
of Schmitz, 434.
of soft sore, 307.
cultivation of, 308.
characters of, 308.
smegmatis, 331.
sporogenes, 556.
subtilis, 396.
group, 732.
suipestifer, 427.
suisepticus, 515.
tertius, 555.
tetani (*see* Tetanus bacillus), 525.
of Timothy-grass (Moeller), 330.
Bacillus, of tuberculosis (*see* Tubercle bacillus), 313.
typhosus (*see* Typhoid bacillus), 406.
in soil, 736.
vesiculosus, 403.
welchii, 545.
cultivation of, 547.
experimental inoculation, 549.
pathogenic effects of, 548.
in soil, 735.
toxigenic properties of, 549.
in water, 739.
whitmori, 361.
of whooping cough, 497.
xerosis, 483.
Y of Fleming, 556.
Bacteria, action of, in nature, 23.
aerobic, 20.
anaerobic, 20.
biology of, 18.
capsulated, 4.
chemical composition of, 11.
classification of, 12, 17.
counting of, in water, 738.
effect of light on, 21.
of moisture on, 19.
food supply of, 18.
higher, 14.
lesions caused by, 158.
lower, 13.
methods of counting, 139, 140, 738.
micro-aerophilic, 20.
microscopic examination of, 96.
modes of division of, 5.
motility of, 8.
movements of, 22.
nitrifying, 26.
nitrogen-fixing, 25.
pyogenic, 235.
relations to oxygen, 19.
to temperature, 20
reproduction of, 4.
spore formation in, 6
staining of, 101.
structure of, 3.
sulphur-containing, 17.
thermophilic, 21.
toxins of, 163.
variability among, 26.
virulence of, 151, 178.
in water, counting of, 737.
Bacteriæmia, 156, 255.
Bacterial action, effects of, 158.
modes of, 155.
ferments, 24.
pigments, 11.
protoplasm, structure of, 9.
treatment of sewage, 745.

Bactericidal action, 195.
effect of radiations, 759.
methods, 129.
powers, natural, 219.
Bacteriolysis, 195.
Bacteriophage, 28.
Bacteriostatic action, 757.
Bacteriotropin, 202.
Bacterium pneumosintes, 494.
tularense, 515.
Bartonella, 706
Basidiomycetes, 710.
Becker's method for spirochætes, 116.
Beggiatoa, 17.
Béraneck's tuberculin, 341.
Berkefeld filter, 87.
Bile-salt media, 58.
Bismarck-brown, 102.
Blackwater fever, 661.
Blastomyces, 727.
Blastomycosis, 726.
Blastomycotic dermatitis, 728.
Blepharoplast, 678.
Blood culture, 145.
film preparations from, 99.
media, 54.
method of obtaining, 145.
samples from rabbits, 133.
Boas-Oppler bacillus, 748.
Boeck and Drbohlav's medium, 670.
Boöphilus bovis, 705.
Bordet and Gengou, bacillus of, 498.
medium of, 56.
reaction of, 134.
Borrelia, 562.
recurrentis, 576.
Botulinus bacillus, pathogenic action of, 541.
Botulismus, 540.
Bouillon media, 40.
Bread paste, 58.
Brilliant green as antiseptic, 759.
enrichment method, 59.
Bromcresol purple, 64.
Broncho-pneumonia, 278.
Bubonic plague, 504.
Buchner's tube, 80.
Bulloch's apparatus for anaerobic culture, 81.
Burke's modification of Gram's method, 108.
Butter bacillus (Rabinowitsch), 330.

Calmette's reaction, 336.
Capsules, staining of, 111.
Carbol-fuchsin, Ziehl-Neelsen, 105.
Carbol-gentian-violet, 106.
Carbol-methylene-blue, 105.
Carbol-thionin-blue, 105.
Carbolic acid as antiseptic, 759.
Carriers, 154
in amœbic dysentery, 668.
cholera, 451.
diphtheria, 476.
of meningococcus, 292.
typhoid, 416.
Casein digest, 41.
Catalase, 20.
Cattle plague, 614.
Centrifuges, 91.
Ceratophyllus fasciatus, 694.
Cerebro-spinal fever, 288.
fluid, examination of, 147.
Chamberland filter, 87.
Chancroid, 307.
Charbon (*see* Anthrax), 380.
symptomatique, 557.
Chemotaxis, 22.
Chicken-pox, 613.
Chlamydobacteria, 16.
Chlamydobacteriales, 18.
Chlamydomucor racemosus, 711.
Chlamydospores, 708.
Chloramine-T as antiseptic, 758.
Chlorine as antiseptic, 758.
Cholera, 445.
carriers, 451.
methods of diagnosis, 458.
preventive inoculation against, 458.
serum reactions in, 456.
Cholera-red reaction, 450.
Cholera vibrio, characters of, 445.
cultivation of, 448.
distribution of, 447.
experimental inoculation, 452.
haemolytic test, 450.
immunity to, 455.
isolation from water, 744.
powers of resistance of, 451.
toxins of, 454.
in water, 741.
Chromidial bodies, 666.
" Chromidial buds," 678.
Chytridiinæ, 710.
Cimex lectularius, 601.
rotundatus, 700.
Cladothrix asteroides, 376.
Clostridium (genus), 544.
botulinum, 540.
chauvœi, 557.
histolyticum, 557.
œdematiens, 555.
œdematis maligni, 551.
sporogene, 556.
tertium, 555.
tetani, 525.
welchii, 545.
Clubs in actinomyces, 16, 367.
Cocci, 2, 13.
Cocco-bacilli, 14.

Coli-typhoid bacilli, agglutination of, 439.
isolation and differentiation of, 437.
group, 397.
Collargol method for spirochætes, 115.
Collodion capsules, 143.
membranes, 611.
Colloidal reactions, 193, 207, 227.
Colonies, enumeration of, 76.
Comma bacillus (*see* Cholera vibrio), 445.
Commensals, 150.
Common colds, 496.
Complement, 196.
in anaphylaxis, 228.
bacteriophilic, 199.
constitution of, 196.
estimation of, 132.
fixation of, 129, 134.
in glanders, 360.
in gonorrhœa, 306.
in tuberculosis, 339.
in whooping-cough, 498.
Congestion, 224.
Conidia, 16.
of actinomyces, 366.
Conidiophore, 708.
Conjunctivitis, 259, 279.
gonorrhœal, 305.
Conorhinus megista, 689.
Conradi and Troch's method for B. diphtheriæ, 60.
Cornet's forceps, 99.
Corrosive sublimate as antiseptic, 756.
as fixative, 119.
Corynebacteria, characters of, 481.
Corynebacterium diphtheriæ, 463.
hofmanni, 482.
xerosis, 483.
Coryza, 496.
Cover-glass films, staining of, 102.
Cover-glasses, cleaning of, 99.
Cowpox, 617.
Crescents, malaria, 652.
Cresol as antiseptic, 759.
Crithidial forms of trypanosomes, 679, 684.
Crystal violet, 102.
as antiseptic, 757.
Culex fatigans, 599.
Culture media, bile-salt, 58.
blood media, 54.
bouillon, 40.
bread paste, 58.
casein digest, 41.
digest bases, 41.
egg media, 56.
glucose agar, 50.
Culture media, glucose broth, 48.
gelatin, 49.
glycerin agar, 50.
broth, 48.
Hartley's broth, 50.
meat extract, 39.
milk, 58.
peptone gelatin, 48.
water, 51.
potato, 57.
preparation of, 39.
reaction of, 42.
Robertson's bullock's heart medium, 51.
serum media, 52 *et seq.*
use of, 67.
Cultures, bacterial, mounting of, 92.
filtration of, 86.
incubation of, 85.
single cell, 72.
Cutaneous reaction in diphtheria, 477.
in scarlatina, 265.
in syphilis, 571.
in tuberculosis, 336.
Cutting of sections, 120.
Cysts of endolimax nana, 667.
of entamœba coli, 667.
histolytica, 666, 667.
iodamœba bütschlii, 667.
Cytase, 196, 217.
Cytolytic sera, 201.

Da Fano's minute bodies, 641.
Dakin-Daufresne solution, 758.
Danysz's bacillus, 429.
Dark-ground illumination, 97.
Decolorising agents, 103.
Deep cultures, 68.
Dehydration of sections, 101.
Delhi sore, 702.
Deneke's vibrio, 460.
Dengue, 599.
Depression immunity, 180.
Dermacentor venustus, 606.
Dermacentroxenus rickettsi, 606.
Dermatitis, blastomycotic, 728.
Dermotropic viruses, 614.
Desensitisation, 225, 231.
Dialister pneumosintes, 495.
Diaplyte antigen, 341.
Dick reaction, 263.
preparation of toxin, 265.
Dientamœba fragilis, 665, 670.
Dieudonné blood-alkali medium, 56.
Digest bases for media, 41.
Diphtheria, 462.
antibodies in, 481.
antitoxin, use of, 479.

Diphtheria, immunity against, 477.
methods of diagnosis, 484.
Schick test, 477.
toxin - antitoxin immunisation, 479.
Diphtheria bacillus, characters of, 463.
cultivation of, 465.
distribution of, 468.
fermentative properties of, 467.
identification of, 481.
inoculation with, 471.
organisms allied to, 481.
associated with, 470.
pathogenic action of, 484.
powers of resistance of, 467.
staining of, 465.
stains for, 109.
toxins of, 472.
virulence of, 475.
tests, 485.
of calves, 376.
carriers, 476.
toxin, preparation of, 473.
standardisation of, 189.
Diphtheroids, 481.
Diplococci, 13.
Diplococcus catarrhalis, 295.
crassus, 296.
intracellularis meningitidis, 288.
mucosus, 296.
pharyngis siccus, 296.
pneumoniæ, 269.
Disaccharides, 62.
Disturbances of metabolism by bacteria, 161.
Dorset's egg media, 56.
Dreyer's diaplyte antigen, 341.
standard agglutination, 126.
Drop method of making dilutions, 123.
Droplet infection, 733.
Drying *in vacuo*, 91.
Ducrey's bacillus, 307.
Durham's tubes, 64.
Dust in relation to infection, 733.
Dysentery, amœbic, 663.
lesions in, 671.
bacillary, 429.
vaccination against, 435.
bacilli, agglutination of, 432.
atypical, 433.
characters of, 430.
immunisation against, 434.
pathogenic properties of, 433.
relations to the disease, 431.
toxins of, 433.
in water, 741.

Eberthella dysenteriæ, 431.
paradysenteriæ, 431.
Eberthella typhi, 406.
Eberth's bacillus (*see* Typhoid bacillus), 406.
Ectodermoses, 614.
Eczema marginatum, 718.
Egg media, 56.
Ehrlich phenomenon, 191.
Ehrlich's rosindol reaction, 66.
side-chain theory, 214.
theory of toxic action, 173.
El Tor vibrio, 457.
Embedding in paraffin, 120.
Encapsulatus pneumoniæ, 286.
rhinoscleromatis, 362.
Encephalitis, epidemic, 640.
experimental transmission of, 642.
lethargica, 641.
of rabbits, 643.
Encephalitozoön cuniculi, 643.
Endocarditis, bacteria in, 25
gonorrhœal, 306.
Endolimax nana, 665, 669.
cysts of, 667.
Endo's medium, 59.
Endospores, 6.
Endotoxins, 164.
Entamœba buccalis, 674.
coli, 663, 665, 668.
cysts of, 667.
experimental infection with, 673.
gingivalis, 674.
histolytica, 663, 665.
characters of, 664.
cultivation of, 670.
cysts of, 666, 667.
distribution of, 671.
experimental inoculation with, 672.
minuta, 664.
nana, 669.
tetragena, 663.
Enterica, 423.
Enterococcus, 240, 242.
Enumeration of colonies, 76.
Epidemic cerebro-spinal meningitis, 288.
encephalitis, 640.
hiccup, 641.
poliomyelitis, 634.
method of investigation, 640.
pathology of, 637.
Epidermophyton inguinale, 718.
Epizootic abortion, 522.
Eppinger's streptothrix, 375.
Erysipelas, 257.
Escherichia, 398.
coli, 403.
communior, 403.
Escherich's bacillus, 403.

Eubacteria, 2.
Eubacteriales, 17.
Eusol, 758.
Exaltation of virulence, 180.
Exotoxins, 166.
Eyre's method of standardising reaction, 43.

Fæces, examination of, 148.
False membrane, 250, 468.
Farcin du bœuf, 376.
Farcy, 354.
Favides, 721.
Favus, 713, 717.
  parasites, cultures of, 718.
Fermentation of sugars, 62.
  tubes, 65.
Ferments adjuvants, 217.
  bacterial, 24, 170.
Fever, 162.
Film preparations, blood, 99.
    dry, 98.
    pus, etc., 99.
    from urine, 100.
    staining of, 102.
    wet, 100.
Filterability of organisms, 611.
Filterable virus of yellow fever, 593.
  viruses, 610.
Filter-passers, 2, 610 *et seq.*
Filter-passing viruses, demonstration of, 614.
Filters, forms of, 87.
  use of, 88, 614.
Filtration of cultures, 86.
Finkler and Prior's vibrio, 460.
Fish, tuberculosis in, 328.
Fixateur, 217.
Fixation of complement, 129, 134.
  of tissues, 119.
Flagella, characters of, 14.
  nature of, 9.
  peritrichous, 14.
  staining of, 112.
Flagellata, 676.
Flaginac reaction, 739.
Flexner-Y bacilli of dysentery, 431.
Flies, contamination of milk by, 752.
  in relation to typhoid, etc., 418.
Flocculation, 125.
  with diphtheria toxin, 189.
  reaction, 572.
    in syhilis, 135, 138.
Fontana's method for spirochætes, 115.
Food-poisoning, 425.
  diagnosis of, 428.
  types of salmonella group associated with, 427.
Foot-and-mouth disease, 610, 613.
Formaldehyde as antiseptic, 759.
Formalin solution as fixative, 119.
Foth's dry mallein, 360.
Fowl pest, 614.
Fraenkel's pneumococcus, 269.
Framboesia, 573.
Friedländer's pneumobacillus, 286.
Fuchsin, basic, 102.
Fungi, diseases produced by, 713.
      immunity and supersensitiveness in, 721.
  imperfecti, 710, 712.
  methods of examination of, 713.
  pathogenic, 707.
Fusiform bacilli, 559.

Gaertner's bacillus, 426.
Gall-stones following typhoid fever, 413.
Gametocytes (malaria), 649, 652.
"Garnet" method for testing antiseptics, 756.
Gas-formation, observation of, 64.
Gas gangrene, 543.
  metastatic, 549.
Geissler's vacuum pump, 87
Gelatin media, 48.
General paralysis, spirochætes in, 568.
    Wassermann reaction in, 572.
Gentian-violet, 102.
Geryk air-pump, 91.
Giemsa's stain, 118.
    method for spirochætes, 116.
Glanders, 353.
  diagnosis of, 361.
  in horses, 353.
  in man, 354.
  serum reactions in, 359.
Glanders bacillus, agglutination of, 359.
    characters of, 355.
    cultivation of, 356.
    inoculation with, 357.
    pathogenic action of, 358.
Globoid bodies in coryza, 496.
    in poliomyelitis, 636, 638.
Globulin constituent in anti-toxin, 210.
Glossina morsitans, 686, 690.
  palpalis, 684.
Glucose media, 48 *et seq*
Glucosides, fermentation of, 62.
Glycerin media, 48 *et seq.*
Goatpox, 618.
Gonococcus, characters of, 299.
  cultivation of, 300.
  fermentation by, 302.
  lesions caused by, 304.
  medium for, 54.
  pathogenic properties of, 303.

Gonococcus, serological types of, 302.
toxin of, 303.
Gonorrhœa, 299.
complement fixation in, 306.
methods of diagnosis, 307.
vaccines in, 306.
Gonorrhœal arthritis, 305.
conjunctivitis, 305.
endocarditis, 306.
Gram's method, 106.
Burke's modification of, 108.
Jensen's modification of, 107.
Much's modification of, 315.
Weigert's modification of, 106.
Granulomata, infective, 161.
Group agglutinins, measurement of, 126.
Gulland's method for fixing sections, 121
Gurd's medium, 54.

Hæmagglutinins, 203.
Hæmamœba, 647.
Hæmatopinus spinulosus, 694.
Hæmoglobinophilic organisms, 488.
Hæmolysin in malaria, 662.
Hæmolysis by streptococci, 241.
Hæmolytic sera, 198.
tests, 131.
Hæmophilus conjunctivitidis, 259.
ducreyii, 307.
influenzæ, 487.
lacunatus, 260.
pertussis, 497.
Hæmosporidia, 647.
Haffkine's vaccine for plague, 512.
Halogens as antiseptics, 758.
Hanging-drop preparations, 70, 96.
Hansen's bacillus (*see* Leprosy bacillus), 346.
Haptophore group, 173.
Hartley's broth, 50.
Heart-water, 601.
Hemispora stellata, 726.
Hemisporosis, 726.
Henry's method of culturing anaerobes, 81.
Herpes, 643.
and encephalitis lethargica, question of relationship, 645.
cell inclusions in, 646.
encephalitis in rabbits due to virus of, 644.
experimental tramsmission of, 644.
zoster, 645.
Herpetic keratitis, 643, 644.
Heterophile antigens, 200.
Higher bacteria, 2.
Hiss's method for staining capsules, 111.
serum water media, 64.
Hofmann's bacillus, 482.
Hog cholera, 614.
"Holder" method for milk, 754.
Horsepox, 618.
Hydrogen-ion concentration, estimation of, 44.
Hydrogen, supply of, 78.
Hydrophobia, 622.
protective inoculation against, 627.
virus of, 625.
Hypersensitiveness, 222.
Hyphæ, 707.
Hyphomycete of mycetoma, 379.
Hypochlorites as antiseptics, 758.
Hypodermic syringes, 141.

Immune-body, 196.
estimation of, 132.
union of, 198.
Immunity, 175 *et seq.*
acquired, 175.
theories as to, 213.
active, 176.
methods of, 177.
artificial, 176.
by dead cultures, 180.
by feeding, 183.
by living cultures, 178.
by sensitised dead cultures, 181
by toxins, 182.
depression, 180.
local, 183.
natural, 219.
passive, 177.
methods of, 178.
unit of, 189.
Incubation of cultures, 85.
Indian ink method for spirochætes, 115.
Indicators, 63.
Indol, formation by bacteria, 65.
production of, by B. coli, etc., 400.
Infant diarrhœa and milk, 752.
Infection, conditions modifying, 151.
Inflammatory conditions, 233 *et seq.*
methods of examination, 267.
Influenza, 486.
filterable virus in, 494.
methods of examination in, 493.
Influenza bacillus, characters of, 487.
distribution of, 490.
experimental inoculation with, 491.

Influenza-like bacilli, 491
Inoculation of animals 77, 141.
Inspissator, 52.
Intermittent sterilisation, 36.
Intracutaneous tuberculin test of Mantoux, 336.
Intraperitoneal injection, 142.
Intraspinal injection, 143.
Intravenous injection, 143.
Involution forms, 5.
Iodamœba bütschlii, 665, 669.
cysts of, 667.
Iodine as antiseptic, 758.

Jaundice, infective, 585.
Jennerian vaccination, 615.
Jenner's strain, 117.
Jensen's modification of Gram's method, 107.
Johne's bacillus, 330.
Joints, gonococci in, 305.

Kala-azar, 695.
Kataphylaxis, 153.
Keratitis, herpetic, 643.
Kinetonucleus, 677, 690
Kinetoplast, 677.
Kipp's apparatus, 79.
Kirkpatrick's, J., method for staining capsules, 112.
for staining flagella, 113.
Klebs-Löffler bacillus (*see* Diphtheria bacillus), 463.
Koch's blood serum, 52.
steam steriliser, 35.
tuberculin, 335, 341.
Koch-Weeks bacillus, 259.
Kühne's methylene-blue, 105.

Lactic acid bacilli, 748.
Lactobacilli Beijerinck, 748.
Lactose fermenters, classification of, 439.
Laverania malariæ, 656.
" Leishman-Donovan bodies," 695.
cultivation of, 698.
Leishmania braziliensis, 703.
donovani, 695.
experimental infection with, 699.
infantum, 701.
tropica, 702.
Leishmaniasis, 694.
methods of examination of, 701.
Leishman's stain, 117.
method for trypanosomes, 677.
Lepra cells, 345.
Leprosy, 344.
bacillus, cultivation of, 349.
distribution of, 347.
Leprosy, diagnosis of, 352.
etiology of, 350.
experimental work, 350.
pathological changes in, 344.
tuberculin reaction in, 351.
Wassermann reaction in, 351.
of rats, 350.
Leptomonas, 699.
Leptospira, 562.
in blackwater fever, 661.
hebdomadis, 598.
icterohæmorrhagiæ, 585.
cultivation of, 586.
inoculation with, 588.
icteroides, 594.
of phlebotomus fever, 598.
Leptospiræ, saprophytic, 589.
Leptothrix, 16, 377.
buccalis, 377.
Lesions caused by bacteria, 158.
by toxins, 161.
Leucocidin, 244.
Leucocytosis, 159.
Levaditi's methods for spirochætes, 114.
Lice in relapsing fever, 581.
in trench fever, 608.
in typhus fever, 603.
Lignières and Spitz's actino-bacillus, 375.
Litmus media, 63.
Liver abscess in dysentery, 672.
Local immunity, 183.
Löffler's bacillus (*see* Diphtheria bacillus), 463.
blood serum, 53.
methylene-blue, 104.
Lower bacteria, 2.
Luetin, 571.
Lumbar puncture, method of, 147.
Lustig's anti-plague serum, 513.
Lysæmia in blackwater fever, 662
Lysogenic action of serum, 195.
towards red corpuscles, 198.

MacConkey's bile-salt media, 58.
use of, in coli-typhoid group, 400, 410, 432.
McFadyean's reaction for anthrax bacillus, 381.
McIntosh and Fildes' anærobic jar, 82.
McLeod's method for culturing anaerobes, 80.
Macrocytase, 217.
Macrogametocytes (malaria), 652.
Macronucleus, 677.
Macrophages, 216.
Madura disease, 377.
Madurella mycetomi, 379.
Malaria, 647.

Malaria, epidemiology of, 658.
experimental transmission of, 659.
hæmolysin in, 662.
immunity in, 660.
malignant, 657.
methods of examination in, 662.
pathology of, 660.
quartan, 656.
relapses in, 654.
sub-tertian, 657.
tertian, 656.
parasites, 648 *et seq.*
cultivation of, 658.
varieties of, 656.
Malignant œdema, 550.
pustule, 390.
Mallein, 360.
Malta fever, 517.
agglutination in, 521.
epidemiology of, 519.
goat's milk as source of infection, 519.
methods of diagnosis in, 521.
Mann's method's for fixing sections, 121.
Marmorek's anti-streptococcic serum, 195.
Mastitis, bovine, 751.
Measles, 632.
serum prophylaxis and treatment of, 633.
virus of, 633.
Meat extract, 39.
Media, tubing of (*see* Culture media). 67.
Mediterranean fever, 517.
Melioidosis, 361.
Meningitic type of poliomyelitis, 639.
Meningitis, bacteria causing, 296.
due to a hæmophilic bacillus, 491.
methods of examination, 297.
tubercular, 322.
Meningococcus, 288.
agglutination of, 294.
carriers of, 292.
diplococci allied to, 295.
distribution of, 291.
experimental inoculation with, 291.
fermentation by, 290.
identification of, 293.
medium for, 55.
types of, 293.
Mercuric chloride as antiseptic, 756.
Mercurochrome-220 as antiseptic, 759.
Mercury compounds as antiseptics, 759.
Merozoites, 648, 649.
Metabolism, disturbances of, 161.
Metachromatic granules, 10.
Metchnikoff's theory of phagocytosis, 216.
vibrio, 459.
Methylene-blue stains, 104.
Methyl-red reaction of B. coli, 401.
for B. coli in water, 739.
Methyl-violet, 102.
Micro-aerophilic bacteria, 20.
Micrococcus catarrhalis, 295.
rheumaticus, 254, 258.
tetragenus, 244.
lesions caused by, 251.
Microcytase, 217.
Microgametocytes, 652.
Micronucleus, 677.
Microphages, 216.
Microscope, use of, 95.
Microscopic methods, 95 *et seq.*
Microspora, 713.
Microsporides, 721.
Microsporon audouini, 714.
furfur, 730.
tardum, 714.
velveticum, 714.
umbonatum, 714.
Microtus montebelli, 599.
Milk as a culture medium, 58.
bacillus coli in, 750, 754.
bacteriology of, 747.
certified, 753.
contamination by flies, 752.
graded, 753.
methods of examination of, 754.
pasteurisation of, 753.
pathogenic organisms in, 751.
souring of, 748.
standards of, 753.
sterilisation of, 753.
tubercle bacilli in, 751, 754.
Mist bacillus (Moeller), 330.
Minimum lethal dose (M.L.D.), 187.
Moeller's grass bacillus, 330.
Molluscum contagiosum, 613.
Monilia candida, 722.
Morax-Axenfeld bacillus, 260.
Mordants, use of, 103.
Morgan's No. 1 bacillus, 435.
Mosaic disease of the tobacco plant 610.
Mosquito, malarial parasites in, 654.
Mosquitoes and malaria, 658.
yellow fever, 592.
Motility, examination for, 96.
of bacteria, 8.
Moulds, media for, 61.

Mounting of bacterial cultures, 92.
Much's method for tubercle bacillus, 315.
Mucor mucedo, 707, 708.
Muir's, Rd., method for staining capsules, 111.
method for staining flagella, 113.
Mumps, 613.
Mutation, 26.
Mycetoma, 377.
Mycobacteria, 314
Mycobacterium avium, 328.
lepræ, 346.
paratuberculosis, 330.
piscium, 328.
smegmatis, 331.
tuberculosis, 313.
tuberculosis bovis, 325.
Mycoderma, 712.
Mycomycetes, 707, 710.
Myxobacteria, 18.
Myxobacteriales, 18.

Nagana, 690.
Naso-pharynx, examination of, 148.
Natural immunity, 219.
Negri bodies, 626.
Negro lethargy, 680.
Neisser and Wechsberg's method, 130.
phenomenon, 197.
Neisseria catarrhalis, 295.
flava, 295.
gonorrhœæ, 299.
intracellularis, 288.
sicca, 296.
Neisser's gonococcus, 299.
stain for diphtheria bacillus, 110.
use of, 465.
Neisser-Wechsberg phenomenon, 197.
Neuroryctes hydrophobiæ, 627.
Neurotropic viruses, 614.
Neutral-red, 64.
action of B. coli on, 400.
Nicolle's medium for trypanosomes, 680.
Nitrates, formation of, 733.
reduction of, by B. coli, 400.
Nitrifying bacteria, 26, 735.
Nitroso-indol method, 66.
Noguchi's medium for leptospira, 587.
tubes for anaerobes, 85.
Noma, 559.
Nordhafen vibrio, 461.
Novy and MacNeal's medium, 679, 698, 703.
Nuclei of bacteria, 9.

Obermeier's "spirillum," 576.
Œdema, malignant, 550.
Oidia, 708.
Oidium albicans, 722.
coccidioides, 729.
lactis, 712, 722.
Oil immersion lens, use of, 95.
Oleate-blood-agar, 489.
Oökinete (malaria), 655.
Oomycetes, 708.
Oospheres, 708.
Oospora lactis, 712.
Ophthalmia neonatorum, 305.
Ophthalmic tuberculin reaction, 336.
Opsonic action, 201.
methods, 127, 128.
Opsonins, 201, 220.
in tuberculosis, 339.
Ornithodorus moubata, 583.
Oroya fever, 706.
Osteomyelitis, suppurative, 256.
Otitis media, 279.
Oxidising agents as antiseptics, 758.
Ozœna bacillus, 363.

Pappataci, 597.
Parabasal body, 697.
Paracholera vibrios, 457.
Paracolon bacilli, 405.
Paraffin, embedding and cutting in, 120.
Parasites, 23, 150.
Paratyphoid bacilli, 423.
preventive inoculation against, 425.
carriers, 424.
Passive anaphylaxis, 225.
immunity, 177, 184, 225.
Pasteurella group, 515.
pestis, 502, 515.
tularensis, 515.
Pasteurisation of milk, 753.
Pasteur's method of exaltation of virulence, 180.
of vaccination against anthrax, 392.
of vaccination against hydrophobia, 628.
Pathogenic fungi, 707.
organisms, 150.
streptothrices, other than actinomyces, 375.
Pediculus capitis, 603.
corporis, 603.
Penicillium, 710.
crustaceum, 712.
glaucum, 712.
Peptone gelatin, 48.
water, 51.

Periostitis, suppurative, 256.
Peritrichous flagella, 14.
Permanganate of potassium as antiseptic, 758.
Pestis major, 506.
minor, 506.
Petri's dishes, 73.
sand-filter method, 731.
Petroff's method for B. tuberculosis, 343.
Pfeifferella mallei, 353.
Pfeiffer's influenza bacillus, 487.
phenomenon, 196.
reaction, 11, 456.
Phagedænic lesions of the genitals, 561.
Phagocytosis, 201.
measurement of, 127.
theory of, 216.
Phenol as antiseptic, 759.
Phlebotomus argentipes, 700.
fever, 597.
flies, 703.
pappatasii, 597.
Phycomycetes, 707.
Pigeon-pox, 614.
Pigments of bacteria, 11.
Pipettes, 122.
Piroplasma bigeminum, 705.
canis, 705.
equi, 705.
parvum, 705.
Piroplasmosis, 704.
Pitfield's method for flagella, 113.
Pityriasis versicolor, 730.
Plague, 501.
bacillus of, 502.
agglutination of, 513.
distribution of, 504.
experimental inoculation, 507.
fleas as vectors of, 510.
bubonic, 504.
immunity phenomena in, 512.
infection in rats, recognition of, 514.
in wild rodents, 511.
lesions in, 504.
methods of diagnosis in, 514.
modes of infection in, 508.
pneumonia, 506.
pneumonic, infection in, 511.
preventive inoculation against, 512.
septicæmic, 506.
vaccine, 512.
Plasmodium, 647.
falciparum, 656, 657.
malariæ, 656.
vivax, 656.
Plasmolysis, 10.
Plate cultures, 73.
Platinum wires, 69.
Pleuro-pneumonia, bovine, 611.
cultivation of virus, 612.
Pneumobacillus, 286.
Pneumococcus, agglutination of, 284.
characters of, 269.
cultivation of, 270.
experimental inoculation, 274.
immunity phenomena, 279.
lesions caused by, 276.
mucosus, 273.
reactions of, 272.
relations to streptococcus, 273.
types of, 283.
Pneumonia, 268.
prophylactic vaccination against, 285.
treatment of, with antisera, 284.
types of, 277.
Polar staining, 10.
Poliomyelitis, epidemic, 634.
Polychrome methylene-blue, 105.
Polysaccharides, 62.
Post-mortems, bacteriological examinations in, 149.
Potato media, 57.
Precipitation, nature of, 207.
Precipitin in pneumonia, 282.
Precipitins, 205.
and anaphylaxis, 227.
Preparation of vaccines, 139.
Protective inoculation, 176 *et seq.*
Proteolytic anaerobes, group of, 545.
Proteosoma, 647.
Proteus capsulatus, 245.
mirabilis, 245.
vulgaris, 245.
X 19, 605.
zenkeri, 245.
Protista, 1.
Protozoal diseases, 647 *et seq.*
Pseudo-diphtheria bacillus, 482.
Pseudomonas aeruginosa, 246.
Pseudospirochætes, 661.
Pseudo-tuberculosis in sheep, 482.
Psittacosis bacillus, 429.
Ptomaine poisoning, 425.
Ptomaines, 163.
Puerperal sepsis, 257.
Pugh's stain for diphtheria bacillus, 110.
Pus, film preparations from, 99.
Pyæmia, 234.
Pyococci, toxins of, 243.
Pyogenic bacteria, 235.
organisms, mode of entrance of, 252.
Pyorrhœa alveolaris, 377, 566, 674.
Pyrogallic acid, use of, 79.

Quarter-evil, 557.
Quinine and blackwater fever, 661.
in malaria, 652, 659, 661.

Rabbits, encephalitis of, 613.
Rabies (*see* Hydrophobia), 622.
antiserum, 630.
methods of diagnosis, 631.
protective inoculation against, 627.
Rabinowitsch's bacillus, 330.
Radiations, bactericidal effect of, 759.
Ramon's flocculation reaction, 189.
Rat-bite fever, 595.
Rattus norvegicus (decumanus), 510.
rattus, 510.
Rat viruses, 429.
Rauschbrand bacillus, 557.
Ray fungus, 364.
Reaction of media, adjustment of, 42.
Receptors as antitoxin, 214.
Reducing agents as antiseptics, 758.
Reduction of nitrates by B. coli, 400.
Red-water fever, 705.
Relapses in malaria, 654.
Relapsing fever, 576.
immunity in, 579.
mode of transmission, 581.
serological reactions in, 579.
spirochætes, cultivation of, 577.
experimental inoculation with, 578.
immunological differentiation of, 584.
varieties of, 580.
Rheumatism, acute, 258.
Rhinitis, 279.
Rhinoscleroma, 362.
Rhizoplast, 697.
Ricin, 171, 183, 190.
Rickettsia bodies, 601.
infections, 601.
melophagi, 602.
prowazeki, 603.
quintana, 609.
rocha-limæ, 604.
Ridcal and Walker's method for antiseptics, 756.
Ring-forms of malarial parasites, 650.
Ringworm parasites, cultures of, 718.
small-spored, 713.
large-spored, 714.
Robertson's bullock's heart medium, 51.
Robin, 171.
Rocky Mountain fever, 606.
Romanowsky stains, 116.
Rosindol reaction (Ehrlich's), 66.
Ross's "thick film process," 662.
Rough and smooth colonies, B. paratyphosus B, 424.
Rous sarcoma, 614.
Roux, antitoxin standard of, 189.
Rust-fungi, 710.

Sabouraud's media, 61.
stain for trichophyta, 118.
Saccharolytic group of anaerobes, 545.
Saccharomyces, 712.
Safranin, 102.
Salmonella enteritidis, 426.
group, 423.
paratyphi, 423.
schottmülleri, 423.
Salt agar, 503.
Saprophytes, 23, 150.
Sarcina lutea, 732.
Sarcinæ, 13.
Sarcoma of Rous, 614.
Scarification, 142.
Scarlatina, 262.
active immunisation, 266.
Schick test, 477.
Schizogony in malaria, 648.
Schizomycetes, 2.
Schizonts, 650.
Schizophyceæ, 3.
Schizophyta, 3.
Schmitz's bacillus, 434.
Schultz-Charlton reaction, 263.
Sclavo's anti-anthrax serum, 393.
Scutula, 717.
Secretion from naso-pharynx, examination of, 148.
Sections, cutting of, 120.
fixing on slides, 121.
Sedimentation, 125.
Seitz filter, 87.
Sensitised vaccines, 181.
Separation of aerobic organisms, 73
Septicæmia, 156, 234.
Septic tanks, 745.
Serum, anti-anthrax, 393.
antibacterial, 194.
antidiphtheritic, 187, 477.
antimeningococcic, 294.
antiplague, 513.
antipneumococcic, 284.
antistreptococcic, 195.
antitetanic, 536.
antitoxic, 187.

Serum, antitubercular, 341.
bactericidal action of, 129.
diagnosis in plague, 513.
disease in man, 230.
hæmolytic, 198.
media, 52 *et seq.*
method of obtaining, 145.
precipitins, 206.
Seven-day fever, 598, 599.
Sewage, bacteriology of, 744.
purification of, 746.
Sheeppox, 614, 618.
Shiga's bacillus of dysentery, 431.
Shmamine's medium, 596.
Side-chain theory, 214.
Single cell cultures, 72.
Sleeping sickness, 680.
lesions in, 681.
leucocytes in, 681.
Sloped cultures, 67.
Smallpox, 615.
antibodies in, 621.
nature of virus, 619.
Smegma bacillus, 331.
Smith, Th., phenomenon of, 224.
Smith-Noguchi medium, 569.
Smith's method for B. diphtheriæ, 60.
Smut-fungi, 710.
Snake venoms, 171.
immunity against, 187.
Sobernheim's anti-anthrax serum, 393.
Soft sore, bacillus of, 307.
Soil bacteria, 735.
bacteriology of, 733.
methods of examination of, 734.
Sonneg roup of dysentery bacilli, 434.
Sore throat, streptococcal, 751.
Specificity of antibodies, 185.
Spirilla, 2, 14.
Spirillum laverani, 597.
minus (*see* Spirochæta morsus muris), 595.
muris, 597.
Spirochæta morsus muris, 595.
Spirochætæ, 2, 14.
Spirochætales, 17.
Spirochætal jaundice, 585.
antibodies in, 588.
antiserum treatment of, 590.
diagnosis by inoculation of blood, 587.
infective, 589.
spirochætes in urine, 588.
Spirochætes, diseases due to, 562.
in Vincent's disease, 560.
staining of, 114 *et seq.*
Spironema (*see* Treponema), 562.
pallidum, 563.
Splenic fever (*see* Anthrax), 380.
Sporangium, 707.
Spore formation, 6.
Spores, staining of, 110.
Sporoblasts, 655.
Sporocyst, 655.
Sporogony, 654.
Sporotrichon beurmanni, 725.
Sporotrichosis, 723.
Sporozoites, 655.
malaria, 648.
"Stab" cultures, 69.
Staining methods, 102.
of bacteria, 101.
of capsules, 111.
of diphtheria bacillus, 109.
of flagella, 112.
of spirochætes, 114 *et seq.*
of spores, 110.
of tubercle bacillus, 109.
principles of, 101.
Stains, basic aniline, 102.
Romanowsky, 116.
Stalactite growth, 504.
Standard agglutinable cultures, 125.
of purity of water, 742.
Staphylococci, experimental inoculation, 247.
lesions caused by, 249.
toxins of, 243.
Staphylococcus, 13.
cereus albus, 237.
flavus, 237.
epidermidis albus, 237.
pyogenes albus, 237.
aureus, 235.
citreus, 237.
tetragenus, 244.
Staphylolysin, 244.
Stegomyia fasciata and yellow fever, 591.
Sterilisation at low temperatures, 37.
by chemicals, 38.
by dry heat, 34.
by filtration, 38.
by heat, 33 *et seq.*
by moist heat, 35.
by steam at high pressure, 36.
of milk, 753.
Sterility, maintenance of, 38.
Stomoxys calcitrans, 638.
"Stormy clot" reaction, 547, 739.
Streptococci, 239.
anaerobic, 240.
experimental inoculation, 248
fæcal, in soil, 735.
fermentation by, 240.
hæmolysis by, 241.
in water, 739.
lesions caused by, 249.

Streptococci, serological classification of, 243
varieties of, 239.
Streptococcus, 13.
anginosus, 240.
brevis, 239.
conglomeratus, 240.
equinus, 240.
erysipelatis, 239, 257.
fæcalis, 240, 259.
lacticus, 748.
longus, 239.
mesenteroides, 13.
mitis, 240.
mucosus encapsulatus, 24.
pyogenes, 237.
salivarius, 240.
saprophyticus, 241.
scarlatinæ, 262.
antitoxic sera, 264.
identity of, 264.
toxins of, 264.
viridans, 241.
Streptothrices, 16.
in actinomycosis, 375.
Streptothrix actinomyces (Bostrom), 371.
(Israel and Wolff), 372.
characters of, 365.
maduræ, 379.
"Stroke" cultures, 69.
Subcutaneous injection, 142.
Substance sensibilisatrice, 196.
Sub-tertian malaria, 656.
Sugars, fermentation of, 62.
Sulphur bacteria, 17.
Summer diarrhœa, 435.
Supersensitiveness, 222 *et seq.*
in glanders, 360.
in tuberculosis, 335.
natural, 231.
Suppuration, nature of, 233.
Suppurative conditions, 233 *et seq.*
Syngamy, 655.
Syphilis, 563.
methods of examination, 573.
serum diagnosis of, 135.
transmission to animals, 569.
Wassermann reaction in, 572.
Syringes, hypodermic, 141.

Tarozzi's method for anaerobes, 85.
Telluric acid medium, 60.
Tertian fever (malaria), 656.
Tetanolysin, 533.
Tetanospasmin, 533.
Tetanus, 524.
experimental, 532.
idiopathic, 531.
immunity against, 536.
methods of examination in, 539.
Tetanus, preventive inoculation against, 539.
Tetanus antitoxin, use of, 537.
Tetanus bacillus, agglutination of, 537.
characters of, 525.
cultures of, 529.
distribution of, 526.
isolation of, 527.
pathogenic action of, 530.
toxins of, 533.
Tetanus dolorosus, 536.
Tetanus toxin, 215.
action of, 534.
Tetrads, 13.
Texas fever, 705.
Theileria parva, 705.
Thermophilic bacteria, 21.
Thiobacteria, 17.
Thiobacteriales, 18.
Thionin blue, 102, 105.
Three-day fever, 600.
Throat swab, 148.
Thrush, 722.
Tick fever, 581.
Timothy grass bacillus (Moeller), 330.
Tinea, 713.
Tobacco plant, mosaic disease of, 610.
Torulæ, 712.
Toxic action, theory of, 172.
Toxin (*see* Special diseases), 473.
estimation of, 187.
preparation of, 187.
standardisation of, 189.
Toxins, 163 *et seq.*
animal, 170.
immunity against, 187.
lesions caused by, 161.
nature of, 168.
of pyococci, 243.
variations in susceptibility to, 221.
vegetable, 170.
Toxoids, 173, 191, 193.
Toxophore group, 173.
Trachoma, 613.
Trench fever, 607.
Treponema, 562.
calligyrum (*see* Treponema gracile), 566.
carteri, 580.
cuniculi, 570.
duttoni, 581.
gallinarum, 584.
gracile, 565.
microdentium, 566.
minutum, 565.
mucosum, 566.
novyi, 580.

Treponema obermeieri, 576.
of yaws, 574.
pallidum, 563.
cultivation of, 568.
dermotropic, 567.
distribution of, 567.
neurotropic, 567.
pertenue, 574.
refringens, 565.
Triatoma megista, 689.
Trichobacteria, 16.
Trichophyta, 714.
Sabouraud's stain for, 118.
Trichophytides, 721.
Trichophyton acuminatum, 715.
crateriforme, 715.
ectothrix, 715.
rosaceum, 715.
violaceum, 715.
Trophonucleus, 677.
Trophozoites (malaria), 648, 649.
Trypagar, 55.
Trypanosoma brucei, 688, 692.
congolense, 693.
cruzi, 687, 689.
equinum, 693.
equiperdum, 693.
evansi, 692.
gambiense, 682, 687.
inoculation with, 683.
lewisi, 693.
melophagium, 693.
rhodesiense, 685, 687, 690.
theileri, 693.
vivax, 693.
Trypanosome fever, 685.
Trypanosomes, infection by, 679.
morphology and biology of, 676.
staining of, 676.
Trypanosomiasis, immunity in, 686.
in animals, 690.
methods of examination in, 688.
serum in, 688.
Tsetse fly disease, 690.
Tubercle bacilli, action of dead, 332.
in milk, 751.
Tubercle bacillus, action on the tissues, 318.
bovine type, 325.
chemical composition of, 315.
cultivation of, 316.
distribution of, 320.
experimental inoculation, 323.
human type, microscopical characters, 313.
lesions caused by, 318.
powers of resistance of, 318.
specific reactions of, 334.
stain for, 109.
staining reactions of, 314.
Tuberculin, 334.
Béraneck, 341.
in diagnosis, 335
Tuberculin-O, 341.
Tuberculin-R, 341.
Tuberculin test, for diagnosis in cattle, 337.
therapy, 338.
varieties of, 341.
Tuberculosis, 311.
agglutination in, 339.
avian, 328.
bovine, 324.
complement fixation in, 339.
immunity phenomena in, 339.
in animals, 312.
in cattle, 337.
in the fish, 328.
methods of examination in, 342.
modes of infection in, 333.
supersensitiveness in, 335.
varieties of, 324.
Tularæmia, 515.
Typhoid bacillus, agglutination of, 420.
biological reactions of, 410.
characters of, 406.
cultivation of, 409.
immunisation against, 414.
in soil, 736.
isolation of, 437.
pathogenic effects of, in animals, 413.
relations to the disease, 415.
suppurations caused by, 412.
toxic products of, 414.
Typhoid carriers, 416, 752.
fever, 397.
epidemiology of, 417.
gall-stones following, 413.
lesions in, 411.
serum diagnosis of, 419.
vaccination against, 421.
Typhoid-paratyphoid group in water, 741.
isolation from water, 743.
Typhus fever, 602.
heterologous agglutinins in, 605.

Ulcerative endocarditis, 254.
experimental, 256.
gingivitis, 560.
stomatitis, 559.
Ultramicroscopic viruses, 1.
(*see* Filter-passing viruses), 1, 610 *et seq.*
Undulant fever, 517.
Undulatory membrane, 677.
Unit of immunity, 189.

Unna's polychrome methylene-blue, 105.
Uredinaceæ, 710.
Urine, examination of, 149.
  film preparations from, 100.
  tubercle bacillus in, 322.
  typhoid bacillus in, 438.
Urobacillus septicus, 245.
Ustilaginaceæ, 710.

Vaccination, Jennerian, 615.
Vaccine bodies, 620.
Vaccines, 178.
  preparation of, 139.
  sensitised, 181.
  treatment by (*see also* Special diseases), 182.
Vaccinia, 617.
  antibodies in, 621.
Variants, "rough" and "smooth," 27.
Variation, 26.
Variola (*see* Smallpox), 615.
Vegetable toxins, 171.
Venins, 171.
Vibrio, 14.
Vibrio choleræ (*see* Cholera vibrio), 445.
  comma, 445.
  Deneke's, 460.
  El Tor, 457.
  metchnikovi, 459.
  nordhafen, 461.
  of Finkler and Prior, 460.
Vibrion septique, 550.
    cultivation of, 553.
    experimental inoculation with, 554.
    immunity to, 554.
Vibrios of paracholera, 457.
Vincent's angina, 559.
  disease, 561.
Virulence, 151.
  attenuation of, 178.
  exaltation of, 180.
Voges and Proskauer's reaction, 400, 442.
Voges-Proskauer reaction for B. coli in water, 739.
Von Pirquet's reaction, 336.

Wart, common, 613.
Wassermann reaction, methods of, 135.
    in general paralysis, 572.
    in leprosy, 351.
    in syphilis, 572.
    in yaws, 575.
Water, bacterial content of, 740.
Water, bacteriology of, 737.
  B. coli in, 738.
  B. dysenteriæ in, 741.
  B. welchii in, 739.
  cholera vibrio in, 741.
  collection of samples of, 737.
  contamination of, by sewage, 741.
  filtration of, 741.
  methods of examination, 737.
  streptococci in, 739.
  typhoid-paratyphoid group in, 741.
Weigert's modification of Gram's method, 106.
Weil-Felix reaction, 605.
Wenyon's modification of Noguchi's leptospira medium, 587.
Wertheim's medium, 54.
Whey agar, 749.
  broth, 749.
Whooping-cough, 497.
  bacillus, characters of, 497.
  fixation of complement in, 498.
  methods of examination in, 499.
Widal reaction, 419.
Winter-spring fevers, 656.
"Woody Tongue," 369.
Woolsorter's disease, 391.
Wound-antiseptics, 758.
Wounds, anaerobes in, 543.
Wright, A. E., bactericidal method, 130.
    blood capsule, 147.
    opsonic method, 128.
    vaccination against tubercle, 339.
    vaccine methods, 139.
Wright's, J. H., stain, 118.
    streptothrix of actinomycosis, 372.

Xerosis bacillus, 483.
Xylol as clearing agent, 101.

Yaws, 573.
Yeasts, 712.
Yellow fever, 590.
    experimental transmission of, 592.
    filterable virus of, 593.
Yersin's anti-plague serum, 513.

Zenker's fluid, 119.
Ziehl-Neelsen carbol-fuchsin, 105.
Zone phenomena, 204, 208.
Zoogloœa, 4.
Zygomycetes, 708.
Zygospore, 708.
Zygote (malaria), 655.