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**AN INTRODUCTION TO
PRACTICAL BACTERIOLOGY**

AN INTRODUCTION TO PRACTICAL BACTERIOLOGY

A Guide to Bacteriological Laboratory Work

BY

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FOURTH EDITION

EDINBURGH
E. & S. LIVINGSTONE
16 & 17 TEVIOT PLACE

1934

to it for those prescribed methods he is called on to use in a practical course or in simple diagnostic work, while the senior student and laboratory worker will find in this part the technical information required for more advanced study. By arranging the text also in large- and small-type matter we have aimed at presenting to the junior worker the essentials of the subject without excessively restricting the scope of the book. We trust that the material added in this edition and the arrangement of the subject-matter will tend to increase the general usefulness of the book.

As in previous editions illustrations have been omitted, except a few diagrams required to facilitate description of apparatus, methods, etc. The book is intended primarily as a guide to bacteriological work and study in the laboratory, where actual apparatus, microscopic preparations, bacterial cultures and other materials are available as the real illustrations of practical instruction.

We are much indebted for many valuable suggestions and information on particular subjects to Professor C. H. Browning, Dr C. P. Beattie, Dr G. M. Findlay, Dr H. J. Gibson, Dr P. Hartley and Dr H. J. Parish, and we have to thank Dr H. A. Ash, Dr H. J. Gibson and Dr D. G. S. McLachlan for their assistance in the reading of proofs and preparation of the index. We would also take this opportunity of acknowledging the helpful criticism and advice of many other colleagues and friends.

T. J. M.
J. E. McC.

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PART I

(INTRODUCTORY)

GENERAL BIOLOGY OF MICRO- ORGANISMS AND IMMUNITY IN RELATION TO PRACTICAL BAC- TERIOLOGY

CHAPTER I

THE GENERAL BIOLOGY OF MICRO-ORGANISMS

THE BIOLOGICAL DIVISIONS REPRESENTED BY THE PATHOGENIC MICROBES

BACTERIOLOGY or Microbiology, as applied to medicine, embraces the study of those micro-organisms which are pathogenic to, or commensals of, man. The term "pathogenic" implies the power of producing disease; organisms that occur on the skin or in certain parts of the body—*e.g.* mouth, throat, bowel—without exerting any harmful effect are described as "commensals." Various commensal organisms are, however, potential pathogens, and some recognised pathogenic microbes may, under certain conditions, assume a commensal rôle—*e.g.* in the so-called infection carriers.

In veterinary science, bacteriology is specially concerned with the micro-organisms responsible for disease in domesticated animals. As many infective diseases are common to man and animals, medical and veterinary bacteriology are closely related branches of the general subject. Pathogenic organisms, however, show great diversity in their parasitism to different animal species, certain being associated with disease in the human species only, while others are highly virulent towards particular animals, though non-pathogenic to man.

The pathogenic and commensal micro-organisms may be classified broadly in the following biological divisions: (1) the bacteria or schizomycetes ("fission

fungi"); (2) the fungi proper, which include the moulds ("hyphomycetes") and the yeasts ("blastomycetes" or "saccharomycetes"); (3) the protozoa.

Certain infective agents, generally regarded as living organisms, are so minute that they can pass through a filter which is impervious to the recognised bacteria, and are designated "filterable viruses." The majority are "ultramicroscopic" (*vide* p. 453) and are still undefined biologically.

The differential characterisation of these biological divisions is as follows:—

BACTERIA.—Simple, unicellular organisms of microscopic dimensions, multiplying with great rapidity usually by simple fission, and dividing in one, two or three directions of space; cells spherical, cylindrical, comma-shaped, spiral or filamentous; devoid of chlorophyll and exhibiting no "morphological" nucleus; certain species develop a resting phase in the form of "spores"; some of the filamentous types produce "conidia" (*vide* p. 9); in certain species the cells are motile and possess flagella; some forms are flexuous.

FUNGI: *Hyphomycete* or *mould* forms.—Branching filaments (hyphae) interlacing and forming a mesh-work (mycelium); more highly organised than the bacteria, often septate and multicellular, and reproduce usually by means of spores developed in "fruiting organs." *Blastomycetes* or *yeast* forms.—Round, oval or elongated cells, generally larger than bacteria and multiply by "budding"; in some species multiple "endospores" formed; and in some, hyphae occur.

PROTOZOA.—Generally regarded as the lowest forms of animal life; minute unicellular organisms with the protoplasm differentiated into nucleus and cytoplasm; reproducing by fission and spore-formation, and often exhibiting a definite life-cycle with both sexual and asexual phases.

The bacteria and the filterable viruses play the most important part in the causation of human infective disease. Protozoal infections are most prevalent in tropical and subtropical countries.

THE BACTERIA

For practical work some scheme of biological classification is necessary. Various systems have been used in the past, but in medical bacteriology a simple classification, with an expressive nomenclature, serves for all practical purposes. Thus, it is generally sufficient to classify the bacteria in the first instance into the following main subdivisions :—

HIGHER BACTERIA

Elongated sheathed filaments, often showing true or false branching; cells may be interdependent, some being specialised for reproduction; more highly organised than the lower bacteria.

Genera of Medical Importance

1. *Leptothrix* — unbranched filaments—e.g. *L. buccalis* (p. 408).
2. *Streptothrix* — filaments which show true branching, and interlace to form a mycelium—e.g. *S. actinomyces* (p. 402).

LOWER BACTERIA (or EUBACTERIA)

Simple unicellular structures never in the form of sheathed filaments; each cell equivalent; many species motile, usually due to their possessing flagella.

Main Morphological Classes

1. Cocci—globose in shape—e.g. the streptococci (p. 252).
2. Bacilli—straight rod-shaped organisms—e.g. *B. typhosus* (p. 328).
3. Vibrios and spirilla—definitely curved non-flexuous rods (vibrios) or spirals (spirilla)—e.g. *V. cholerae* (p. 359).
4. Spirochaetes—flexuous spiral filaments—e.g. *Treponema pallidum* of syphilis (p. 411)

MORPHOLOGICAL STUDY OF THE BACTERIA

UNSTAINED PREPARATIONS. — The morphology of the bacteria can be studied by examining them first in the unstained condition, suspended in fluid. In this way their general shape can be observed and motility determined. Certain very slender organisms, however, such as the spirochaetes, are so feebly refractile that they cannot be seen by the ordinary microscopic method, and *dark-ground illumination* (*vide* p. 58) is necessary for their demonstration.

STAINED PREPARATIONS. — The examination of stained preparations is usually an essential routine procedure. For this purpose various aniline dyes are employed, often along with a mordant. For the demonstration of spirochaetes "*negative staining*" has been used—*i.e.* where the organism is mixed with some substance such as Indian ink, which, in film preparations, yields a dark background, while the organism stands out clear and unstained. *Silver impregnation* methods (*vide* p. 171) are also utilised for the routine demonstration of spirochaetes.

STAINING REACTIONS. — The staining reactions of the bacteria are of the greatest importance both in their morphological study and for their differentiation and identification. Thus, all the bacteria can be divided into two categories by the so-called Gram's staining reaction (*vide* p. 152)—*i.e.* according to whether they resist decolorisation with aniline oil, alcohol or acetone after staining with a pararosaniline dye—*e.g.* gentian or methyl violet, and subsequent treatment with iodine. Those retaining the dye are designated "Gram-positive"; those decolorised by this method are spoken of as "Gram-negative."

It is supposed that the reaction depends on the permeability of the bacterial envelope. The iodine forms a compound with the dye in the protoplasm of the organism. This compound is insoluble in water but soluble in the decoloriser, and is retained in the cells of Gram-positive bacteria by virtue of the impermeability of the envelope, whereas it is more readily removed from the Gram-negative types.

Some bacteria, when treated with a strong staining solution, resist decolorisation by acid, and are spoken of as "acid-fast"—e.g. the tubercle bacillus (*vide* p. 158). This property is generally regarded as due to a constituent of lipoidal nature which is characteristic of these organisms.

Certain organisms do not stain uniformly and this may be a characteristic feature. Thus the diphtheria bacillus shows a "beaded" or "barred" appearance when stained with methylene blue. The plague bacillus displays "bipolar staining," the ends being more deeply coloured than the centre.

A staining reaction which is characteristic of certain species—e.g. *B. diphtheriae*—is the appearance of metachromatic granules—i.e. certain granules are demonstrated in the bacterial protoplasm, which can be stained with one dye while the rest of the protoplasm stains with a dye of different colour (*vide* p. 160). These structures are sometimes called "volutin" granules. They exhibit a special affinity for nuclear stains and with polychrome methylene blue are coloured purple. They are apparently rich in nucleoprotein but do not represent nuclear elements, and are probably not vital constituents of the bacterial protoplasm. Their occurrence and number depend on the medium in which the organism is growing.

PLEOMORPHISM AND INVOLUTION.—It must be remembered that, especially in artificial culture, bacteria may show considerable variation in shape and size (pleomorphism), and may also exhibit degeneration or "involution" forms which are different morphologically from the normal cell.

MOTILITY.—The motility of bacteria (observed in a fluid medium) is generally due to delicate prolongations of the protoplasm (flagella) which act as locomotory organs. These are not seen in unstained preparations and can be demonstrated only by special staining methods (*vide* p. 164). Brownian movement must not be confused with true motility. In the latter case the organism definitely changes its position in the

microscope field (*vide* p. 57). Brownian movement is generally regarded as due to the impact of the molecules of the medium in which the organisms are suspended and is an oscillatory movement within a limited radius.

Flagella may be "terminal"—*i.e.* at one or both ends of the bacterium—and single or multiple. They may be distributed all round the organism, and this arrangement is described as "peritrichate."

When there is a single terminal flagellum the term "monotrichate" is applied; "amphitrichate" indicates the presence of a flagellum at each pole; "lophotrichate" refers to the arrangement of multiple flagella at one or both poles.

Among the spirochaetes motility is generally due to contractions of the cell body. Though certain spirochaetes have been described as possessing flagella-like structures, these organisms are regarded as characteristically devoid of flagella.

BACTERIAL SPORES.—Some species develop a highly resistant resting-phase, or spore, by which the individual survives unfavourable external conditions. The spore is not a reproductive structure. In the vast majority of spore-bearing species only one spore is developed by each vegetative form. This structure appears in the cell protoplasm ("endogenous") and increases in size, appearing as a round, oval or elongated body, which may be situated in the centre of the bacterium ("central"), at the end ("terminal"), or between the centre and end ("subterminal"). The relative size of the spore varies with different species. Spores can withstand all injurious chemical and physical influences better than the vegetative forms, and owe certain of their resistant properties to a dense outer protective membrane. Under favourable external conditions the membrane ruptures and the vegetative form is resumed. This process is described as the "germination" of the spore.

The spore is not stained by the ordinary methods but appears as a clear, unstained portion of the bacterial

structure. Special methods are employed for the differential staining of spores (*vide* p. 162).

BACTERIAL CAPSULES.—Certain bacteria may exhibit a relatively thick outer capsule and are described as “capsulated.” The formation of this “capsule” depends on environmental conditions (*vide* pneumococcus, p. 265). Carbohydrate substances enter into the composition of such capsules, and may be of great importance in determining certain specific characters of bacteria (*vide* p. 29). By ordinary methods of staining the capsule appears as an unstained zone round the organism. Special methods are available for the differential staining of capsules (*vide* p. 163).

THE BACTERIAL PROTOPLASM.—No structure can be defined readily in the bacterial protoplasm corresponding to a nucleus. In all probability bacteria possess nuclear material, but either it is scattered in granular form throughout the cytoplasm or the nuclear apparatus exists in some form incapable of exact demonstration by existing methods. It is of interest that the cell protoplasm exhibits an affinity for those basic aniline dyes which are used as nuclear stains.

MULTIPLICATION AMONG THE BACTERIA.—Among the lower bacteria multiplication takes place by simple binary fission. The cell enlarges or elongates, and a constriction develops transversely which ultimately divides the original individual into two new cells. Division may occur with great rapidity—*e.g.* every half-hour—so that one individual may soon reproduce several millions of new organisms. Among the spirochaetes transverse fission occurs as in other bacteria.

In the higher bacteria transverse division of the filaments into shorter forms is observed. Certain filaments also divide up at their free end into a number of oval “conidia,” which are set free, and each of these, besides representing a resting-phase, may develop a new colony.

Some observers have described more complex processes of reproduction among bacteria, and have regarded the usual

forms which multiply by simple fission as one phase only in a life-cycle which they postulate. The evidence at present does not warrant acceptance of such views.

COCCHI

Cocci are classified morphologically as follows :—

Staphylococcus.—The individuals tend to be grouped in clusters, due to the irregularity of the planes of successive divisions.

Streptococcus.—The cocci are arranged in chains, successive divisions being always in the same axis.

Tetracoccus.—Plates of four (or multiples of four) cocci, division occurring successively in two planes at right angles.

Sarcina.—Packets of eight (or multiples of eight) cocci, division occurring successively in three planes at right angles.

Diplococcus.—The cells tend to be arranged uniformly in pairs.

The different cocci are relatively uniform in size, 1μ (0.001 millimetre) being the average approximate diameter. Some species are capsulated. Shape varies with species, being spherical, oval, lanceolate, or kidney-shaped. The reaction to Gram's stain is an important criterion in their identification.

BACILLI

Morphological features of importance in the study and identification of these organisms are :

Size—some being relatively large—e.g. *B. anthracis* (p. 313), others small—e.g. *B. influenzae* (p. 378).

Shape—rectangular—e.g. *B. anthracis*; oval (coccobacilli)—e.g. *B. pestis* (p. 365).

Arrangement—in pairs—e.g. pneumobacillus (p. 326); in chains—e.g. *B. anthracis*.

Occurrence of a capsule—e.g. pneumobacillus.

Motility, flagella and their arrangement—e.g. *B. typhosus* possesses numerous flagella which are peritrichate in arrangement.

Spores, their shape and position—e.g. *B. tetani* is characterised by its spherical terminal spore.

Reaction to Gram's stain (p. 157).

Acid-fastness—e.g. tubercle bacillus.

Staining of cytoplasm—e.g. "beading," bipolar staining, metachromatic granules (*vide supra*).

The bacilli cannot be classified readily into morphological genera, and physiological characters have to be taken into account in their classification. In the past the term "bacillus" has been used as a generic name for all the straight rod-shaped forms, but it must be admitted that such use of the term can hardly be justified in the strict biological sense in view of the great diversity of species which exhibit this morphological form.

VIBRIOS AND SPIRILLA

Comma- and S-shaped forms and non-flexuous spirals are characteristic (*vide supra*). Most species are very actively motile (e.g. "darting" motility) and the flagella are terminal. They are mostly Gram-negative.

While there is no sharp line of demarcation between the vibrios and spirilla, these organisms have usually been classified into two morphological genera: *vibrio*—the typically short curved rod-shaped form; and *spirillum*—the spiral filamentous type.

SPIROCHAETES

Some spirochaetes are relatively large, refractile and easily stained by ordinary methods—e.g. *Borrelia* (or *Spironema*) *refringens* (p. 417); others are delicate, feebly refractile and difficult to stain—e.g. *Treponema pallidum*. The coils may be regular corkscrew-like spirals or only irregular curves. Movement is undulating or rotatory. Though the ends taper and may show "terminal filaments," these are not true flagellar structures. It has been stated that in certain pathogenic species granules are developed in the protoplasm and extruded from the cell, and these are regarded as a phase in the life history of the organism.

The pathogenic forms can be classified broadly into the following genera :

- (1) *Borrelia* (or *Spironema*)—coils are large and wavy, of relatively long wave-length (*e.g.* 2–3 μ) and generally three to seven in number. These organisms are usually more refractile and more readily stained by the ordinary aniline dye solutions than the other pathogenic spirochaetes. They are exemplified by the relapsing fever spirochaetes (*vide* p. 418) and certain commensal species, such as *Borr.* (or *Sp.*) *refringens* (*vide* p. 417).
- (2) *Treponema*—coils are of shorter wave-length (*e.g.* 1 μ), generally regular, sometimes relatively rigid, the organism presenting typically a “corkscrew-like” structure. These spirochaetes are usually feebly refractile and difficult to stain by the ordinary staining solutions. *Tr. pallidum* exemplifies this genus (*vide* p. 411).
(Some systematists classify all the spirochaetes included in these two genera into one composite genus, designated *Treponema*.)
- (3) *Leptospira*—sharply twisted filaments with one or both extremities “hooked” or recurved; the coils are fine, closely wound and numerous, and may be difficult to demonstrate except by dark-ground illumination. Spirochaetes of this group, like the *Treponemata*, are feebly refractile and difficult to stain by the ordinary methods. They differ from the other genera in being able to survive in distilled water. *L. icterohaemorrhagiae* of infectious jaundice is an example of the genus (*vide* p. 421).

NEWER CLASSIFICATION AND NOMENCLATURE

A system of classification and nomenclature of the bacteria (or schizomycetes) was introduced some years ago by the Society of American Bacteriologists following strictly the accepted rules of biological classification, and has since been elaborated by American systematists. A brief outline of this

system is given here, but only those orders, families and genera which are of special importance in medical and veterinary bacteriology are dealt with. The object of this outline is merely to enable the new grouping and nomenclature to be correlated with the older system. Detailed generic characters are not given, but these are indicated in a general way by the various type species quoted, the characters of which are described in later chapters. It should be noted that this system of classification is based on physiological as well as morphological characters. For full details, the third edition of Bergey's *Manual of Determinative Bacteriology* should be consulted.

ORDERS OF BACTERIA (OR SCHIZOMYCETES)

1. **EUBACTERIALES**.—Undifferentiated simple forms, without true branching—spherical, rod-shaped or spiral—some motile, due to flagella—non-flexuous—multiplying by transverse fission—some produce endospores—include many pathogenic species.

2. **ACTINOMYCETALES**.—Differentiated forms, occurring as spheres, rods, spirals or elongated filaments—the longer forms may show branching—endospores not produced—conidia may be formed—the order includes a number of important pathogenic species.

3. **SPIROCHAETALES**.—Usually slender flexuous spiral forms—in some species multiplying by longitudinal fission—the order includes certain important pathogenic species.

- | | | |
|--|---|--------------------------------------|
| 4. Myxobacteriales
5. Thiobacteriales
6. Chlamydobacteriales | } | Include no known pathogenic species. |
|--|---|--------------------------------------|

EUBACTERIALES

Families :—

NITROBACTERIACEAE.—Includes no pathogens.

COCCACEAE.—Spherical or globose organisms.

SPIRILLACEAE.—Spiral forms—fission transverse—non-flexuous.

BACTERIACEAE.—Straight rod-shaped organisms—non-sporing.

BACILLACEAE.—Like the Bacteriaceae but producing spores.

COCCACEAE

Genera of special importance :—

Neisseria.—e.g. meningococcus, gonococcus, etc. (p. 271).

Gaffkya.—e.g. *M. tetragenus* (p. 260).

Diplococcus.—e.g. pneumococcus (p. 265).

Streptococcus (p. 252).

Staphylococcus (p. 249).

Micrococcus.—Occurring in plates or masses of cocci—Gram-positive—some produce yellow pigment—type species is *M. luteus*—saprophytes and facultative parasites.

Sarcina.—Cells arranged in regular packets (p. 10).

SPIRILLACEAE

Genera :—

Vibrio.—e.g. *V. cholerae* (p. 359).

Spirillum.—Rigid spiral organisms—forming long curves or portions of a turn—some motile with multiple polar flagella—not easily cultivated artificially—found in water.

BACTERIACEAE

Genera of special importance :—

Pseudomonas.—e.g. *B. pyocyaneus* (p. 261).

Escherichia.—e.g. *B. coli communis* (p. 322).

Aerobacter.—Like *Escherichia*, but forming acetyl-methyl-carbinol (Voges-Proskauer reaction)—e.g. *B. lactis aerogenes* (p. 325).

Proteus.—e.g. *B. proteus* (p. 261).

Salmonella.—e.g. *B. enteritidis* Gaertner (p. 339).

Eberthella.—e.g. *B. typhosus* (p. 328).

Shigella.—e.g. *B. dysenteriae* (p. 345).

Alcaligenes.—e.g. *B. faecalis alkaligenes* (p. 348).

Brucella.¹—e.g. *B. melitensis* (p. 372).

Klebsiella.—e.g. *pneumobacillus* (p. 326).

Pasteurella.—e.g. *B. pestis* (p. 365).

Haemophilus.—e.g. *B. influenzae* (p. 378).

Dialister.—e.g. *Bacterium pneumosintes* (p. 381).

Lactobacillus.—e.g. *B. acidophilus* (p. 357).

Bacteroides.—e.g. *B. bifidus* (p. 358).

BACILLACEAE

Genera :—

Bacillus.—e.g. *B. anthracis* (p. 313).

Clostridium.—e.g. *B. tetani* (p. 384).

ACTINOMYCETALES

Families :—

ACTINOMYCETACEAE.—Filaments, often branched and forming mycelium—conidia sometimes produced.

MYCOBACTERIACEAE.—Rod-shaped, rarely filamentous—only occasional branching—no conidia.

¹ Some systematists have classified *B. melitensis* and its immediate allies along with *Alcaligenes*, but it has become customary now to include these organisms in a separate genus designated *Brucella*.

ACTINOMYCETACEAE

Genera :—

Actinobacillus (p. 406).

Leptotrichia.—e.g. *Leptothrix buccalis* (p. 408).

Actinomyces (p. 402).

Erysipelothrix.—e.g. *Bacillus* of swine erysipelas (p. 409).

MYCOBACTERIACEAE

Genera :—

Mycobacterium.—e.g. *B. tuberculosis* (p. 297).

Corynebacterium.—e.g. *B. diphtheriae* (p. 282).

Fusiformis.—e.g. *B. fusiformis* (p. 409).

Pfeifferella. e.g. *B. mallei* (p. 310).

SPIROCHAETALES

Genera of special importance :—

Borrelia.—e.g. *Borr. recurrentis* (p. 418).

Treponema.—e.g. *Tr. pallidum* (p. 411).

Leptospira.—e.g. *L. icterohaemorrhagiae* (p. 421).

PHYSIOLOGY OF THE BACTERIA

The physiology and biochemistry of bacteria can be studied by observations made with “cultures” growing in the laboratory in a nutrient medium.

Bacteria are subject, as regards their growth and vitality, to various external influences—e.g. atmosphere, temperature and moisture of their environment, light, chemicals, etc.

ATMOSPHERE.—Some species grow most abundantly in the presence of *free* oxygen—i.e. when exposed to air—and are described as “aerobes”—e.g. *B. anthracis*. Certain bacteria, in fact, are “obligatory aerobes,” their growth being arrested in the absence of free oxygen. Others fail to grow in the presence of *free* oxygen (“obligatory anaerobes”—e.g. *B. tetani*). Evidence has been obtained by certain observers that in the presence of free oxygen these organisms form hydrogen peroxide which is inhibitory to their growth, and unlike certain other peroxide-forming bacteria do not produce catalase (*vide* p. 136). The majority of pathogens are, however, indifferent as regards atmospheric

conditions and will flourish both in the presence or absence of oxygen ("facultative anaerobes"—e.g. *B. typhosus*). Certain pathogenic bacteria grow best in the presence of a trace only of *free* oxygen and are designated "micro-aerophilic"—e.g. one type of the *Streptothrix actinomyces*. An atmosphere with a certain concentration of carbon dioxide (5–10 per cent.) is essential for the growth of the bovine type of *B. abortus* when first isolated from the body.

Recent studies have shown that bacterial respiration depends on complex oxidative mechanisms, the consideration of which is beyond the scope of this book. For further information on the subject, reference should be made to one of the larger works.

TEMPERATURE.—(a) *Influence on Growth*.—For each species there is a definite temperature range within which growth takes place. The limits are the "maximum" and "minimum" temperatures, and an intermediate "optimum" temperature can usually be recognised at which the best growth occurs. The optimum temperature of a bacterium is approximately that of its natural habitat—i.e. about 37° C. in the case of the micro-organisms pathogenic to man. Some organisms have a wide temperature range (e.g. 5°–43° C.), others are more restricted (e.g. 25°–40° C.). Below the minimum, viability is not necessarily interfered with, but above the maximum death more or less quickly ensues, except in spore-bearing species.

(b) *Influence on Viability*.—Heat is an important agent in the artificial destruction of micro-organisms, the effect depending on coagulation of cell-proteins. In general, among the bacteria which are parasites of mammalian animals, non-sporing forms cannot withstand temperatures above 45° C. for any length of time.

The "*thermal death-point*" is the lowest temperature (above the maximum for growth) which kills a particular organism in a given time—e.g. ten minutes.

Thus the thermal death-point of the pneumococcus is approximately 52° C.

Bacteria are more susceptible to moist than dry heat. Spores are much more resistant to heat than vegetative bacteria, but their degree of resistance varies in different species. Further data on this subject are given in regard to sterilisation (p. 67).

Some species die if kept at 0° C., but others may survive even lower temperatures.

MOISTURE.—Four-fifths by weight of the bacterial cell consist of water, and, as in the case of other organisms, moisture is necessary for growth. Drying is generally injurious; spores, however, can resist this influence for long periods—*e.g.* spores of *B. anthracis*, for several years. Certain non-sporing bacteria may also exhibit considerable resistance to drying—*e.g.* the tubercle bacillus (*q.v.*).

LIGHT.—The optimum condition for growth and viability is darkness. Ultra-violet rays are markedly bactericidal—*e.g.* direct sunlight or radiation from an arc or mercury-vapour lamp.

INFLUENCE OF CHEMICALS.—Various inorganic and organic chemicals, acting as protoplasmic poisons, are either inhibitory to growth or bactericidal, depending on the concentration brought into contact with the particular organism.

Substances possessing marked growth-inhibitory or bactericidal properties are usually designated *antiseptics*, and their practical applications are now well known. A great variety of chemicals exhibit antiseptic action and have been used for that purpose: *acids*—*e.g.* nitric acid; *alkalies*—*e.g.* caustic soda; *metallic salts*—*e.g.* perchloride and biniodide of mercury; *organic metallic compounds*—*e.g.* mercuriochrome, silver salvarsan; *halogens*—*e.g.* chlorine (as derived from bleaching powder, etc.), iodine; *alcohols*—*e.g.* ethyl alcohol; *aldehydes*—*e.g.* formalin; *cyclic hydrocarbons*—*e.g.* benzol; *cyclic alcohols*—*e.g.* phenol, cresol; *volatile oils and their products*—*e.g.* thymol; *anaesthetic substances*—*e.g.* chloroform;

oxidizing agents—e.g. hydrogen peroxide; *reducing agents*—e.g. sulphurous acid; various *aniline dyes*—e.g. brilliant green, acriflavine, etc.

It should be noted that even neutral salts, such as sodium chloride, may exert growth-inhibitory or bactericidal properties in certain concentrations.

ELECTRICAL INFLUENCES.—Bacteria may be killed by the passage of an electrical current through the medium in which they are contained. It is doubtful whether this is due to a direct electrical effect. In the case of an alternating current, the resultant heating effect may be bactericidal *per se*, and, if a direct current is used, electrolytic changes with the liberation of acid, nascent oxygen or chlorine may lead to bactericidal effects of a chemical nature.

FOOD SUPPLY.—The growth of bacteria is, of course, dependent on an adequate supply of suitable food material. This varies with the natural adaptations of different species. Thus, as a general rule, in the artificial culture of the pathogens the medium should approximate to the composition of the tissues and body fluids (*vide* p. 75). Certain bacteria can be cultivated in a variety of food media; others are highly restricted in their special food requirements. This subject is dealt with in detail from the practical standpoint in Chapter IV. The H-ion concentration of the medium is also an essential factor in influencing growth (*vide* p. 89). The majority of bacteria grow best at a slightly alkaline reaction.

MUTUAL INFLUENCES.—Different bacteria may flourish well together, the presence of one species favouring the growth of another—*symbiosis*—e.g. staphylococci favour the growth of *B. influenzae*. The reverse effect may also be observed—*antibiosis*—where one species is antagonistic to another—e.g. *B. pyocyaneus*, to various other bacteria.

BACTERIAL ENZYMES.—In the metabolism of bacteria and in the biochemistry of bacterial action enzymes play an essential part. One of the great functions of bacteria in nature is to produce chemical decomposi-

tion of complex organic substances—*e.g.* proteins and carbohydrates—by means of their enzymes. Among the pathogens, fermentative properties are important features in the identification of certain species. Thus, various bacteria decompose particular carbohydrates (*e.g.* sugars, hexahydric alcohols, polysaccharides) with the formation of acids (*e.g.* formic, acetic and lactic acids) and, in many cases, gases (*e.g.* carbon dioxide and hydrogen). Certain species are also capable of breaking down salts of the organic acids.

Some bacteria possess marked proteolytic properties and digest complex protein substances, such as gelatin, coagulated serum, etc., liberating the various products of protein disintegration—*e.g.* amino-acids, ammonia compounds, indole, skatole, hydrogen sulphide, etc. Certain species, while unable to digest complex proteins, act on peptone with the production of indole, etc.

Some bacteria, including certain pathogens, are CHROMOGENIC, and produce characteristic pigments, which in some cases diffuse readily from the bacterial cells into the surrounding medium.

GROWTH PHASES.—When bacteria grow in a fluid medium, their development proceeds as follows: there is first a period of “lag” (*e.g.* two hours) during which there is no increase in number or only slow multiplication; this is followed by a phase of rapid multiplication (*e.g.* during seven to eight hours at optimum temperature); the increase in number is in geometric progression and the logarithmic curve of growth is a straight line; this is called the “logarithmic” phase; then follows a “stationary” phase, multiplication gradually ceasing; in this phase (lasting from a few hours to several days) the number of new individuals tends to be equalled by those dying; finally there is a phase of decline or diminution of viable individuals, the organisms progressively dying until (after several days to months) no living bacteria remain. It should be noted that the initial “lag” phase may be absent when fresh medium is inoculated with organisms which are in the phase of multiplication.

BACTERIAL TOXINS.—These are defined as the products of bacteria which are injurious to the tissues and in

virtue of which disease processes result from bacterial infection. They have not been isolated as chemically pure substances, but are generally regarded as being of protein nature. They are classified as :

1. Extracellular toxins, or exotoxins, which diffuse readily from the bacteria into the surrounding medium.
2. Intracellular toxins, or endotoxins, which are retained within the cells until the bacteria die and disintegrate.

The majority of the pathogens produce endotoxin only, but certain develop powerful exotoxins—e.g. *B. diphtheriae*, *B. dysenteriae* (Shiga), *B. tetani*, *B. botulinus*, certain types of staphylococci and streptococci.

Exotoxins are generally unstable substances, their toxic effect being annulled readily by chemicals, free oxygen, and heat—e.g., in the case of the diphtheria toxin, at 65° C. They tend to be specific in their action on particular tissues. Preparations of these toxins can be obtained by growing the bacteria in fluid culture which is then filtered through an earthenware or other bacterial filter—the filtrate contains the toxin (*vide* pp. 73, 284). After introduction into the body there is usually a short incubation period (e.g. some hours) before symptoms of poisoning are manifest. By immunising animals with such preparations a specific neutralising antibody (anti-toxin) is developed which can be demonstrated in the blood serum of the immune animal (*vide* p. 27).

Most pathogenic organisms do not produce specific diffusible toxins, and their culture-filtrates are non-toxic ; on the other hand, their dead bodies are toxic, and it is assumed that their poisons are bound up in the bacterial cell—hence the designation endotoxin.

These toxins are less specific in their action and produce a more general poisoning effect. They are more stable than exotoxins and can withstand a temperature of 100°C. They can be demonstrated experimentally by injecting dead cultures or bacterial extracts into animals. There is no incubation period following

their introduction as in the case of the exotoxins. Immunisation with endotoxins does not lead usually to the formation of antitoxin.

Certain toxins are specially designated according to their particular effects, irrespective of the organism producing them—*e.g.* haemolysins (producing lysis of red blood cells), leucocidins (destructive to leucocytes).

Some pathogenic bacteria neither produce exotoxin nor are their dead bodies definitely toxic—*e.g.* *B. anthracis*. It is probable that such organisms can produce their toxins only when growing in the tissues. Many pathogenic bacteria, especially when growing in the tissues, form substances which increase their aggressiveness (*vide infra*), and this type of product has been designated "*aggressin*." It is supposed that it acts mainly by interfering with phagocytosis, and that by its agency bacteria are able to break down the first defences of the tissues. Various bacterial products, however, may possess aggressin-like properties, and the existence of a single specialised substance of this type is doubtful.

VIRULENCE OF MICRO-ORGANISMS.—Virulence has generally been regarded as an important property of micro-organisms in relation to their pathogenic action, and is defined as the capacity to invade the tissues, multiply and produce toxic effects. Virulence is estimated by the *minimum lethal dose*—*i.e.* the smallest dose of the organism (*e.g.* in the form of a culture) which will kill a particular species of animal. As a result of the varying susceptibility of individual animals to bacteria and their toxins, it is often impossible to state the exact minimum dose, and it is now customary to refer to the *average lethal dose* for a number of animals. In other parts of the book the symbol M.L.D. (minimum lethal dose) may be taken to denote the average lethal dose. It must be noted that virulence depends on two factors which may be quite independent of one another: the invasive power or aggressiveness, and the toxigenic property of the organism.

The virulence of an organism can be either "exalted" or "attenuated" artificially.

Exaltation of virulence may be produced by passing the strain through a series of individuals of the same species, inoculating the animals one from another in series—*i.e.* "passage." The virulence is increased for the particular species.

Attenuation usually results when organisms are cultivated artificially for some time; thus the stock laboratory cultures are usually of low virulence, as compared with recently isolated strains. Other methods of lowering virulence are referred to on p. 27.

VARIATION AND DISSOCIATION.—Bacterial cultures may, under certain conditions, "dissociate" and develop variants which differ in certain respects from the original strain—*e.g.* morphological features, absence of motility, loss of capsules, the appearance of colonies on culture medium, fermentative properties, virulence, antigenic characters as evidenced by serological reactions, etc. (*vide* p. 34). The variant strain may retain the new feature or property as a stable character. Such dissociation is met with in various groups but has been specially investigated among the Gram-negative bacilli occurring as commensals or pathogens in the intestine (*vide* Chapter XIV.). For example, a strain of coliform bacillus may at first fail to ferment lactose (*vide* p. 325) but develop a lactose-fermenting variant. In colonies on solid media such variants may appear as small papillae. Variation in the physical characters of colonies may also be noted in pure cultures of certain organisms—*e.g.* the "rough" (R) and "smooth" (S) colonies seen in the typhoid-paratyphoid and other groups. These differences in colony-characters are associated with differences in virulence, antigenic constitution and immunising properties. Thus, the rough (R) variant of the pneumococcus is avirulent and this variation is associated also with loss of capsule-formation. Motile bacteria may develop non-motile variants which differ in antigenic characters from the motile type. The relationship of these variations to antigenic characters is dealt with later in connection with agglutinating antibodies (*vide* p. 34). For further information on this subject one of the larger works should be consulted.

BACTERIOPHAGE ACTION or TRANSMISSIBLE LYSIS*(Twort-d'Herelle Phenomenon)*

This phenomenon was demonstrated by d'Herelle in the following way: a few drops of liquid faeces from a case of bacillary dysentery were added to a tube of bouillon which was incubated overnight; filtration of the culture through an earthenware filter yielded a filtrate which, added in small quantities to a young culture of *Bacillus dysenteriae* (Shiga), produced lysis of the bacteria after a period of incubation; further, a filtrate of this lysed culture possessed a similar lytic property towards a fresh culture, and the lytic property was shown to be thus indefinitely transmissible from culture to culture. If a filtrate containing bacteriophage is added to a young broth culture and this is used for inoculating a plate of medium, the resulting growth shows clear circular plaques where the phage is acting; these have been spoken of as "colonies of bacteriophage." It has been suggested by d'Herelle that the effect is due to an ultramicroscopic organism (*Bacteriophage*) which is parasitic and destructive towards the particular bacteria, propagating itself at their expense. It is believed by others, however, that the lytic agent is an enzyme developed by the bacteria themselves and that the lytic property once acquired becomes transmissible in culture. The phenomenon appears frequently when bacteria are growing in contact with tissues. The lytic principle has considerable resistance to physical and chemical agents; it may withstand heating at 75° C. for half-an-hour, and resists drying for long periods. It can be propagated only in living and growing cultures. No definite statement can be made as to whether the phenomenon is due to a living organism or not. The question of the unity or multiplicity of bacteriophages is still unsettled: a bacteriophage which is active for one organism may lyse related bacteria and may apparently be "adapted" to entirely different organisms. Bacteriophage has an antigenic individuality apart from the associated organism and immunisation with phage-containing filtrates yields an antiserum which inhibits the action of the phage.

SYSTEM OF IDENTIFICATION OF THE BACTERIA

1. THE MORPHOLOGY, TOGETHER WITH THE STAINING REACTIONS, OF INDIVIDUAL ORGANISMS generally serves as a preliminary criterion, particularly for placing an unknown species in its appropriate biological group. In medical bacteriology the microscopic characters of

certain organisms in pathological specimens may be sufficient for diagnostic identification—*e.g.* tubercle bacilli in sputum. Morphology among the bacteria usually fails, however, to differentiate allied organisms—*e.g.* meningococcus, gonococcus, *Micrococcus catarrhalis*.

2. CULTURAL CHARACTERS, OR THE MORPHOLOGY OF GROWTHS ON ARTIFICIAL MEDIUM—*e.g.* the appearances of “colonies” to the naked eye or with certain magnifications. This criterion is important in identification, but may also be insufficient to differentiate species—*e.g.* different species of the typhoid-paratyphoid-dysentery groups produce indistinguishable colonies.

3. PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERS—*e.g.* the fermentation of various carbohydrates. Species which cannot be distinguished by morphology and cultural characters may exhibit marked differences in their biochemical reactions—*e.g.* typhoid-paratyphoid group. Different species may, however, resemble one another closely in fermentative properties—*e.g.* *B. paratyphosus* B and *B. enteritidis* Gaertner.

4. SEROLOGICAL TESTS.—In bacteriology, species and types can often be identified accurately by specific “antibody reactions.” These depend on the fact that the serum of an animal immunised against a micro-organism contains specific antibodies (for the homologous species) which react in a characteristic manner with the particular organism (antigen) (*vide* p. 30). Such antisera, for example, may agglutinate or clump the homologous organism in test-tube experiment, and this effect can be observed easily with the naked eye.

It may also be noted here that the serum of a person suffering from a bacterial infection may agglutinate the particular organism, and in this way the agglutination reaction can be used for diagnostic purposes—*e.g.* the Widal reaction in enteric fever, etc.

5. ANIMAL EXPERIMENT. —In the case of pathogenic organisms—*e.g.* *B. tuberculosis*—which are virulent to,

and produce characteristic lesions in, laboratory animals, the inoculation test provides a reliable method of identification.

FUNGI; PROTOZOA

The general morphology and physiology of these organisms need hardly be dealt with here. The biological aspects of the groups that are of special importance in practical bacteriology as applied to medicine will be referred to later in the consideration of particular organisms (Chapters XXI. and XXII.).

FILTERABLE VIRUSES

Little is yet known regarding the biology of these viruses. It is usually assumed that they represent living organisms, owing to the fact that they can be propagated through a series of animals. The majority, though demonstrable as viruses by the experimental method, have not been observed microscopically, and the term "ultramicroscopic" has also been applied to them. Exceedingly minute filterable organisms, however, have been demonstrated microscopically and even cultivated artificially—e.g. *Bacterium pneumosintes* (*vide* p. 381), and the organism of bovine pleuropneumonia.

These viruses are easily destroyed by injurious physical and chemical agencies but as a rule are more resistant than the non-spore-forming bacteria. They often possess a high degree of resistance to glycerol.

Their demonstration and identification depend on the experimental production of a characteristic pathological condition in animals (or man) by means of filtrates after subjecting material from the particular disease (e.g. nasal washings, serum) to filtration through an earthenware filter (*vide* p. 456).

The subject of the filterable viruses is dealt with in further detail in Chapter XXIII.

CHAPTER II

IMMUNITY IN RELATION TO PRACTICAL BACTERIOLOGY

THE subject of immunity is intimately related to practical bacteriology, and immunological principles underlie certain bacteriological methods.

The term "immunity," in its usual application, signifies the power of the animal body to resist (1) infection by parasitic microbes,¹ or (2) the injurious effects of their products or toxins.

Immunity may be *acquired*, as in the natural recovery from infection, and is due to the development, during the illness, of *specific* resisting powers against the causal organism¹ or its toxin. Once developed, acquired immunity may persist for long periods, even practically throughout life, as exemplified by the immunity following smallpox. On the other hand it may be transient—*e.g.* after pneumonia.

An acquired immunity may also be developed artificially by inoculating an animal with a micro-organism¹ or a toxin in such altered state that it is incapable of reproducing the typical disease though still able to bring about an immunity reaction. Such immunity is described as an *active artificial immunity*.

The blood serum of an actively immunised animal injected into the body of a non-immune animal renders the latter temporarily immune, and this is termed *passive immunisation*.

The most frequent methods of producing an active artificial immunity are :

¹ The terms "microbe," "micro-organism" and "organism" used in this chapter on immunity apply also to the viruses (*vide* p. 453).

1. Introduction of living organisms, in a state of attenuated virulence. Various methods have been used to reduce virulence for purposes of immunisation—*e.g.* (1) subjecting them to drying, as in Pasteur's method of attenuating the rabies virus (present in the spinal cord of experimentally infected rabbits); (2) cultivating at a temperature above the optimum, as in the case of Pasteur's anthrax vaccine; (3) passage through animals of a different species; thus vaccinia virus (as used for smallpox vaccination) may be regarded as a form of the smallpox virus attenuated for the human species by its propagation in bovines.
2. Introduction of organisms—usually in the form of cultures—killed by heat or antiseptics—*e.g.* the usual form of bacterial vaccine. Repeated and increasing doses are administered. This system is applied in the prophylaxis of certain infections (*e.g.* anti-typhoid vaccination), in therapeutic immunisation (*e.g.* treatment of chronic infections with vaccines), and also in the preparation of antibacterial sera in animals. Viruses killed by antiseptics—*e.g.* phenol—have also been used for immunisation.

In immunising animals these two methods may be combined; the process may be started with dead organisms, followed by doses of attenuated living organisms, and completed by injecting graded doses of virulent living organisms. In some cases, immunisation is carried out by giving non-lethal doses of unaltered living organisms, without previous injection of dead or attenuated organisms, but this method may be difficult to control.

3. Introduction of toxins in repeated and increasing doses, starting with small and harmless doses and graduating the series, so that, as the immunity progressively develops, each dose is devoid of harmful effect. This is exemplified

in the preparation of diphtheria and tetanus antitoxins by immunising animals—*e.g.* horses—with the respective toxins. To ensure that no injurious effects may result from immunisation, toxin which has been rendered non-toxic (toxoid) by certain chemicals—*e.g.* iodine, formalin, etc.—or by heat, or has been neutralised by antitoxin, is frequently used. Immunisation with diphtheria toxin has been applied in the prophylaxis of diphtheria in the human subject, but to obviate any harmful results it is necessary to introduce it along with antitoxin (toxin-antitoxin immunisation); toxoid is now generally used in preference to toxin (*vide* p. 289).

The serum of an actively immunised animal is designated an *immune serum* or *antiserum*, and owes its effect to specific *antibodies* or *immune bodies* which act adversely on the homologous organism or neutralise its toxins.

The special principle in the organism or its toxin which incites antibody production is termed an *antigen*.

A serum containing antibodies antagonistic to the particular bacterium is spoken of as an *antibacterial serum*; one which contains antibodies that neutralise toxin, as an *antitoxic serum*.

It has to be noted that, apart from bacteria and their toxins, other cells, and in fact all soluble complete protein substances, may act as antigens and incite specific antibody production—*e.g.* red blood corpuscles, blood serum, animal venoms, etc.

Thus the red corpuscles of one species injected into an animal of different species incite the development of an antibody which, under certain conditions, can effect lysis of the red cells of the former. This antibody is described as a *haemolysin* and the serum containing it as a *haemolytic antiserum*.

As a general rule, an antigen, to produce immunity, must be injected parenterally—*i.e.* by some route

other than the alimentary tract (subcutaneously, intravenously, etc.).

Usually an animal can be immunised only against an antigen which is foreign to its own tissues. Thus, a rabbit can be immunised against ox or sheep red cells, but not against the cells of its own species.

Certain non-protein substances—*e.g.* lipoids, carbohydrates, etc.—though unable to act as antigens *in vivo*,¹ may when combined with protein be capable of inciting the formation of antibodies which *in vitro*¹ react specifically with the non-protein substance—*e.g.* in the precipitin or complement-fixation reaction (*vide* p. 30). To these substances the term *haptén* is applied. The specific characters of the antigen of a bacterium may depend on a non-protein constituent—*e.g.* a polysaccharide as in the case of the different types of the pneumococcus (*vide* p. 269).

Specificity is one of the pronounced characters of acquired immunity and of antibodies, and is usually for the biological species, as in the case of *B. typhosus* (*vide* p. 329), though in certain cases it may be more restricted—*e.g.* to “types” within the supposed species, as in the case of the serological types of the pneumococcus (*vide* p. 267).

It must be remembered that, in some instances, the occurrence of antibodies may have no aetiological significance. Thus, in typhus fever a serum-antibody is demonstrable which is specific for a particular type of *B. proteus*—though this organism has no aetiological relationship to the disease (*vide* p. 429).

Specific antibodies may also occur naturally: thus, the serum of the guinea-pig contains a haemolytic antibody for ox red corpuscles; specific bactericidal and agglutinating antibodies (*vide infra*) for various bacteria may be demonstrated in the serum of normal animals.

An antitoxic serum is produced by immunising an animal with exotoxin. For example, “diphtheria anti-

¹ “*In vivo*” denotes “in the living body”; “*in vitro*” (literally, “in glass”) means “in test-tube experiment.”

toxin " is the serum of a horse which has been immunised with graded doses of diphtheria toxin. When appropriate amounts of toxin and antitoxin are mixed together the mixture is non-toxic. This process of neutralisation is complex and need not be dealt with here. It occurs both *in vivo* and *in vitro*.

The antitoxic principle is associated with the globulins of the serum; by a process of precipitating the globulin with half-saturated ammonium sulphate solution, separating the precipitate, dialysing out the ammonium sulphate, and then redissolving the precipitated globulin in a quantity of physiological salt solution much less than the original volume of the serum, an antitoxic serum can be "concentrated" and "refined."

Endotoxins unlike exotoxins do not as a rule incite the production of antitoxins.

Antibacterial sera are generally produced by immunising with the actual bacteria, or bacterial extracts.

These sera may exhibit the following properties :—

1. *Bacteriolytic* or *bactericidal*—*i.e.* directly destroying the bacteria.
2. *Opsonic* or *bacteriotropic*—*i.e.* rendering the bacteria susceptible to phagocytosis.
3. *Agglutinating*—immobilising and clumping the bacteria.
4. *Precipitating* or producing a precipitate along with the soluble products of the bacteria.
5. *Complement-fixing*—*i.e.* along with the bacterial antigen "fixing," "deviating" or "absorbing" complement, a normal constituent of serum (*vide infra*).

These effects may be demonstrated *in vitro*. They are all relatively specific for the particular organism and are quantitatively marked—*i.e.* they are produced by exceedingly minute amounts of the antiserum.

The serum of a person or animal infected with pathogenic bacteria may exhibit also similar properties and

constitutes an antibacterial serum. Thus the serum of a person suffering from typhoid fever exhibits these properties when tested *in vitro* with the typhoid bacillus.

It has to be noted that normal serum may possess bactericidal, bacteriotropic and agglutinating effects. These are relatively weak as compared with the corresponding effects produced by an immune serum.

The bactericidal action of an immune serum is due to a specific thermostable¹ antibody (*bacteriolysin* or *bactericidin*) acting along with a normal non-specific constituent of serum (*complement*) which is thermolabile² (at 55° C.). The antibody apparently combines firmly with the antigen (the bacteria) and the complement then unites with the combined antigen and antibody. The antibody and complement have no independent combining affinity. It has thus been supposed that the antibody acts by "sensitising" the bacteria to the action of complement, and that the latter is the essential lytic agent.

Thus:

Bacteria + { Specific
antibody } + Complement = Bactericidal
effect.

Bacteria + { Specific
antibody } = No effect.

Bacteria + Complement = No effect.

Owing to the original idea that the antibody acted as a combining link between the antigen and complement, the term "amboceptor" has been applied to it.

In vivo bacteriolysis can be demonstrated readily by *Pfeiffer's reaction*: a suspension of cholera vibrios is injected intraperitoneally in a guinea-pig along with an anticholera serum, and if peritoneal fluid be drawn off with a hypodermic syringe after half-an-hour and after

¹ "Thermostable" denotes the ability to withstand a certain degree of heat.

² "Thermolabile" indicates that a particular property is lost on exposure to a certain degree of heat.

one hour, it is seen that the vibrios undergo progressive lysis and disappear from the fluid. In this case the complement of the blood plasma of the animal acts along with the antibody of the immune serum.

Bactericidal action by a normal serum, in many cases, is analogous to that produced by an immune serum, and is dependent on a natural antibody (*vide supra*) and complement. Normal bactericidal effects towards various Gram-positive bacteria are independent of complement and are due to a thermostable (55° C.) principle whose exact nature is still undetermined.

Haemolysis by a haemolytic antiserum is analogous to bacteriolysis—*i.e.* it is due to a specific thermostable antibody acting along with the normal complement.

Thus:

Red Cells + { Specific antibody }	+ Complement = Haemolysis.
Red Cells + { Specific antibody }	= No effect.
Red Cells + Complement	= No effect.

The phenomenon of haemolysis by serum can be demonstrated *in vitro* with blood suspensions and is easily visible with the naked eye, the blood becoming laked or transparent.

A suspension of red blood corpuscles in isotonic salt solution *plus* the antiserum which has been heated at 55° C. to annul complement (*i.e.* red cells + specific antibody only) serves as a test for the presence or absence of complement—*e.g.* in complement-fixation tests—and is spoken of as a *haemolytic system* (*vide p. 35*).

The opsonic effect of normal serum is produced by a non-specific thermolabile principle, the *normal opsonin*.

The increased opsonic action of an antibacterial serum has been regarded as due to a specific thermostable antibody (*immune opsonin*) which can be differentiated from the normal opsonin.

The opsonins combine with the bacteria rendering them in some way susceptible to phagocytosis but without directly affecting their viability. Thus, if a serum is allowed to interact with organisms, which are then separated from it by centrifugalisation and washing with salt solution, they are still susceptible to phagocytosis by leucocytes though the serum has been removed and the leucocytes have also been freed from serum. The opsonin is apparently "absorbed" from the serum by the organisms and bound by them.

The *opsonic index* is a numerical expression of the opsonic power of the serum of a person for a given organism as compared with normal, and has been regarded as significant of the degree of resistance possessed by the person to the particular infection. The index can be estimated according to the following system: a preparation of leucocytes, the bacteria in question and the patient's serum are mixed, and, after a period of incubation, film preparations are made from the mixture and suitably stained; by counting under the microscope, the average number of bacteria phagocytosed by one leucocyte is calculated—the *phagocytic index*. A similar experiment is carried out, substituting the pooled serum of two or three normal persons, and the phagocytic index again estimated. The opsonic index is then calculated by dividing the phagocytic index of the serum in question by that of normal serum. Thus unity is normal, and the opsonic power of the serum is greater or less than normal according as the index is greater or less than unity.¹

Agglutination is the most easily observed effect of an antibacterial serum. If the serum is added *in vitro* to a uniformly turbid suspension of the particular organism and the mixture is incubated, the bacteria become aggregated in clumps and the suspension appears flocculent or granular, the clumps or floccules being easily visible to the naked eye. The phenomenon is attributed to a specific antibody designated *agglutinin*. The agglutinin does not affect the vitality of the bacteria. It also clumps dead bacteria in the same

¹ See Wright, *Technique of the Teat and Capillary Glass Tube*, London, 1921; *Studies in Immunity*, 1909.

way as the living organisms. It is relatively thermostable—*e.g.* as compared with complement. Salts (electrolytes) are necessary for its action and agglutination tests are usually carried out in a medium of physiological saline. The physics of bacterial agglutination is not yet fully understood but depends on a disturbance in the balance between the cohesive force of surface tension tending to aggregate the bacterial cells and the mutually repellent influence of the similar electrical charges carried by the cells. The electrolyte present as well as the agglutinin contributes to the physical changes involved in the process of agglutination. Agglutination tests are applied in diagnostic work, and for the identification of species and types (*vide* p. 24).

AGGLUTINATION REACTIONS IN RELATION TO ANTIGENIC VARIATION.—In laboratory cultures of the typhoid-paratyphoid-dysentery bacilli two types of colony may be observed: (1) the normal smooth, round and transparent form—S (smooth) type, and (2) a rough, irregular and opaque variant—R (rough) type. The S type when suspended in 0.85 per cent. saline forms a stable suspension; the R type tends to auto-agglutinate, unless in weaker saline solutions (*e.g.* 0.2 per cent.). A similar variation with the development of R colonies is seen in various other bacterial groups.

Among motile species two different types of agglutinin (*i.e.* the antigen which stimulates agglutinin formation) can be identified: (a) "flagellar" (contained in the flagella) and "somatic" (in the body of the organism). The former reacting with an antiserum yields "large-flake," the latter "small-flake" or granular agglutination. These different types of antigen have been designated "H" (flagellar) and "O" (somatic) and it has been shown that their thermostability differs: the H antigen is generally labile at 80°–100° C., the O resists 100° C. On the other hand, the O-agglutinin is labile at 70° C. whereas the H-agglutinin is stable at this temperature.

The variation from "smooth" to "rough" is associated with a change in the somatic antigen, the variant antigen being designated by the symbol Ø (or R); the H antigen is unaltered.

Some bacteria possess a single specific H antigen (*e.g.* *B. typhosus*); others are "diphasic" in this respect and their H antigen may occur in two forms, one highly specific, the

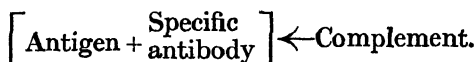
other common to heterologous species. This is well illustrated by *B. paratyphosus B* and related organisms (*vide p. 343*).

These facts summarise briefly the variations in antigenic composition which have to be reckoned with in the practical application of the agglutination phenomenon for diagnostic purposes. Further references to this question are made later (*vide pp. 201, 342*). For more detailed information on the subject one of the larger text-books should be consulted.

The precipitating effect of an antibacterial serum is analogous in many respects to agglutination and is spoken of as being due to an antibody called *precipitin*.

Complement plays an essential part in the bactericidal action of an immune serum, and is absorbed by the bacteria *plus* bactericidal immune body. Even in the absence of bactericidal action, an immune serum may contain antibodies which, along with antigen, fix, deviate, or absorb complement; and a *complement-fixing antibody* is therefore spoken of.

Thus:



To test for this effect the haemolytic system is used as an indicator: if complement has been fixed, then on adding the haemolytic system no haemolysis will occur (*vide p. 32*).

Complement-fixation tests are employed in diagnosis, and occasionally for the identification of species in the same way as the agglutination reaction.

The various immune properties of an antibacterial serum have been described above in terms of multiple antibodies, each designated according to its effect on the antigen (bacteriolysin, agglutinin, opsonin, precipitin, complement-fixing antibody), but it is still an open question whether these reactions are actually due to separate principles in the serum or are merely different manifestations of the activity of a single antibody.

The serum of a person or animal immunised against a virus may exhibit properties which are analogous

to those of an antibacterial serum. Thus, the virus is specifically inactivated by the serum, and this has been spoken of as due to a "viricidal" antibody, the term implying the killing of the virus. The actual effect of this antibody on the virus has not been fully elucidated. Specific precipitating and complement-fixing reactions have also been observed with antiviral sera.

ANAPHYLAXIS AND HYPERSENSITIVENESS

Under certain conditions the parenteral injection of alien protein leads to a *specific* sensitising effect so that the subsequent injection of the same substance may produce toxic and even fatal results.

Thus, if a guinea-pig is injected subcutaneously with horse serum (even an extremely small dose—e.g. 0.001–0.01 c.c.), and after an interval of ten days receives a larger second dose of the same serum (e.g. 0.2 c.c. intravenously or 5 c.c. subcutaneously), it may develop a sudden illness or *anaphylactic shock* in which the chief manifestation is spasmodic constriction of unstriped muscle, particularly that of the small bronchi. The serum may be quite non-toxic *per se* when given to an unsensitised animal even in large doses.

Substances that lead to anaphylaxis (*anaphylactogens*) are those which act as antigens in relation to immunity, and the phenomenon of anaphylaxis is regarded as due to the *in vivo* interaction of antigen and antibody (*precipitin*). Passive hypersensitiveness can also be conferred by injecting the serum of a sensitised animal into a normal animal.

The results of anaphylaxis depend on the mode of injection of the substance into the sensitised animal and the quantity introduced. Thus, anaphylactic shock is more liable to occur and is more marked when the injection is intravenous or intraspinal, and when a large dose is given, than when the injection is subcutaneous or a small quantity is introduced. Thus a dose which would produce shock if given at once,

when introduced in small fractions may not lead to an anaphylactic reaction and the animal is "desensitised" in this way. The state of hypersensitiveness, once developed, may persist for long periods. If, after the sensitising injection but before sensitiveness has developed, a second dose of the substance is given, the animal is protected for a time against a subsequent injection—i.e. *anti-anaphylaxis* has been developed.

Haptens may elicit anaphylactic shock in specifically sensitised animals.

The nature of these phenomena is still obscure. Various theoretical explanations have been advanced on the basis of experimental data. For further details one of the larger works should be consulted. Serum anaphylaxis has, of course, become of practical importance in medicine in relation to serum therapy—for example, when it is necessary to give a second dose of a therapeutic serum after an interval, and especially by intravenous injection; anaphylactic shock must, in such cases, be obviated by desensitisation with very small doses of serum (*vide supra*).

Such hypersensitiveness can be tested for by injecting intracutaneously (*vide p. 257*) 0.1 c.c. of the serum. A positive result is denoted by the occurrence within thirty minutes of an urticarial reaction at the site of inoculation, which may develop a vesicle and may be surrounded by a wide erythematous zone. Hypersensitiveness can also be demonstrated by instillation of serum into the conjunctival sac, or by applying it to a small scarified area of skin.

Bacterial antigens when injected in the form of vaccines rarely give rise to any appreciable hypersensitiveness.

Serum sickness or serum disease.—It should be noted that after a single dose of foreign serum (e.g. a therapeutic antiserum from the horse) given for the first time, in a considerable proportion of normal persons toxic effects may ensue. These occur after an incubation period of eight to twelve days, and consist of fever, an erythematous or urticarial eruption, swelling of lymph glands and joints, albuminuria, etc., and

there may be an inflammatory reaction at the site of the injection. These are regarded as due to a natural hypersensitiveness to the horse serum. It is still doubtful whether this condition is related to anaphylaxis or to the so-called "atopy" which is referred to later.

Hypersensitiveness to the products of the infecting organism (*allergy*) is a feature of various infections in man and animals, and can be demonstrated by the general and local reaction manifested on injection of preparations from cultures of the particular organism—*e.g.* the tuberculin reaction (*vide* p. 303). In such cases the sensitiveness can also be elicited by simple cutaneous or intracutaneous tests. This form of hypersensitiveness differs from anaphylaxis in certain respects. Thus, it is doubtful whether such allergy is dependent on a serum antibody, and it has not been generally possible to transfer the sensitiveness to a normal individual by injection of serum.

Atopy.—In certain persons as a result of genetic factors, hypersensitiveness may occur towards a considerable variety of substances of antigenic nature, so that when the person is exposed to contact with the substance to which he is sensitive, toxic effects result—*e.g.* coryza, asthma, urticaria, gastro-intestinal disturbance, etc. This form of sensitiveness has been designated *atopy*, and is responsible for such conditions as hay fever, asthma, etc. Substances to which such sensitiveness can be attributed (*atopens*) are: plant pollens (as in hay fever), dandruff of animals (*e.g.* horse), proteins of various articles of food, etc. Atopy can be tested for by cutaneous reactions with preparations of the particular atopen, as in testing bacterial allergy. While atopy cannot be transferred in the same way as anaphylaxis, it has been found that when the serum of an atopic person is injected into the skin of a non-sensitive person, and after an interval the atopen is injected at the same site, an urticarial wheal results. The serum therefore contains some active substance (designated *reagin*). This principle, however, differs in certain respects from antibodies.

PART II

BACTERIOLOGICAL TECHNIQUE

**MICROSCOPY; CULTIVATION OF BACTERIA;
STAINING METHODS; ANIMAL INOCULA-
TION AND AUTOPSY; IMMUNOLOGICAL
AND SEROLOGICAL METHODS; BACTERIO-
LOGICAL EXAMINATION OF WATER AND
MILK; AND TESTING OF ANTISEPTICS**

CHAPTER III

THE USE OF THE MICROSCOPE IN BACTERIOLOGY

MICROSCOPY is of primary importance in bacteriology, and a suitable microscope and a knowledge of its use are essential to those engaged in bacteriological work. To obtain satisfactory results, the microscope should be used under the best possible conditions, and the worker must know also the limitations of the instrument.

The component parts of the microscope are as follows :—

The *eye-piece* fits into a graduated *draw-tube*, which in turn slides in the *body-tube*. The lower end of the tube is furnished with a *revolving nose-piece* (preferably “triple”), into which are screwed the *objectives*. The tube and objectives are moved up and down by means of a rack and pinion termed the *coarse adjustment*, while the fine movements necessary for accurate focussing are performed with the *fine adjustment*. The tube and adjustments are supported by an *upright* which is connected to the *foot* by means of a *hinged joint*. Attached to the upright near the joint is a platform called the *stage*, on which is placed the microscopical preparation to be examined. In the centre of the stage is an aperture through which the object can be illuminated from below. A *mechanical stage* may be fitted either to the stand or the fixed stage, so that the preparation can be moved about horizontally, thus ensuring steady and controlled movement. Below the stage is the *substage*, which should be furnished with a means of raising or lowering—usually

a rack and pinion or a spiral screw. The substage is fitted with a *substage condenser*, attached to which lies the *iris diaphragm*. Fitted to the end of the *tail-piece* is a *mirror*, mounted on a gimbal fitting. The plane side of the mirror is employed when a condenser is used; the concave side is used only in the absence of a condenser, and the focal length is such that light comes to a focus on the object examined.

The draw-tube should be adjusted to the *tube length* (*vide* p. 44) for which the objective is corrected; for the majority of microscopes this is usually 160 mm., but as the revolving nose-piece has a length of 18 mm. the draw-tube should be extended only to the 142 mm. mark. Leitz objectives, however, are corrected for a tube length of 170 mm. It is essential that an objective should be used at its proper tube length, particularly the apochromatic objectives (*vide infra*), if the maximum resolution is to be obtained.

The working of the *fine adjustment* varies according to the make of the microscope, for different manufacturers have their own particular type of mechanism. The older forms were actuated by means of a milled head mounted on a pillar behind the coarse adjustment, which turned a screw with a fine thread. Newer models have a milled head parallel to the coarse adjustment. The movements of the milled head raise or lower the tube in the same direction as the coarse adjustment. The milled head is graduated in $\frac{1}{100}$ ths, and one division corresponds usually to a movement of the tube of 0.002 mm. The manner of securing a fine movement is by a system of either levers, cams or cogwheels.

The fixed *stage* should be large enough to take a 4 in. Petri dish, and should be fitted with two clips. A *mechanical stage* is of great advantage, and is particularly useful when a large area of a microscopic preparation has to be searched, as in the examination of films of sputum for tubercle bacilli, or of blood for malaria parasites. An attachable mechanical stage works satisfactorily and can be obtained for almost any model. The "built-in" mechanical stage is more costly, but is to be recommended for routine bacterio-

logical work, as it is steadier and not likely to get out of order.

The *substage* is an important part of the microscope and one to which, unfortunately, little attention is paid. The mechanism of raising the substage should be rigid and free from lateral movement. Where critical work has to be done with highly corrected objectives and condenser, centering screws should be fitted. The *condenser*, which is used for focussing light on the object to be examined, is usually of the two-lens Abbe type; but if apochromatic or semi-apochromatic objectives are used, a condenser of similar optical quality should be employed. The *iris diaphragm* is an important part of the substage, as it controls the amount of light passing into the condenser.

Binocular microscopes which can be used with oil-immersion objectives are now in common use. The rays of light from the objective are divided by a half-silvered surface inclined at an angle of 45 degrees which permits one half of the light to pass vertically, while the remainder is reflected horizontally. Each half of the rays is directed into its appropriate eye-piece by means of prisms. The eye-piece sockets can be adjusted to the interocular distance of the observer, while the left ocular tube is adjustable to correct individual differences of vision between the two eyes.

These binocular microscopes have interchangeable monocular and binocular bodies which are removable without disturbing the objectives. The field is flat, and objects appear somewhat larger than with the monocular body. Pseudo-stereoscopic effects may be obtained, and isolated organisms are more easily recognised. These microscopes are very pleasant to work with and save a considerable amount of eye fatigue. They are particularly useful where much microscopic examination has to be done—as in the routine examination of sputum for tubercle bacilli. We strongly recommend their use for all routine bacteriological and histological work.

OBJECTIVES AND EYE-PIECES

For general purposes, ordinary achromatic objectives are quite satisfactory, and are admirable for routine work and students' use. The quality of the present-day objective is extremely good, and for ordinary work the purchase of the more expensive types is not recommended. The most useful objectives are $\frac{2}{3}$ -in. or 16 mm., $\frac{1}{2}$ -in. or 4 mm., and the $\frac{1}{1\frac{1}{2}}$ -in. (thus designated, but actually $\frac{1}{1\frac{1}{4}}$ -in.) oil immersion. These should be used in conjunction with a 10-magnification ($10\times$) Huyghens eye-piece. A $5\times$ eye-piece is often supplied, and is sometimes employed for searching when a larger field is desired without altering the objective. It is not practicable to use the ordinary (Huyghens) eye-piece above $12\times$, and even this magnification gives some distortion and haziness of outline. Higher magnifications may be obtained by the use of apochromatic objectives and compensating eye-pieces. With binocular microscopes $6\times$ or $8\times$ eye-pieces are sufficient, as, owing to the division of the rays, less light enters each eye-piece. With the $10\times$ eye-piece the field is apt to be too dark when ordinary illuminants are used.

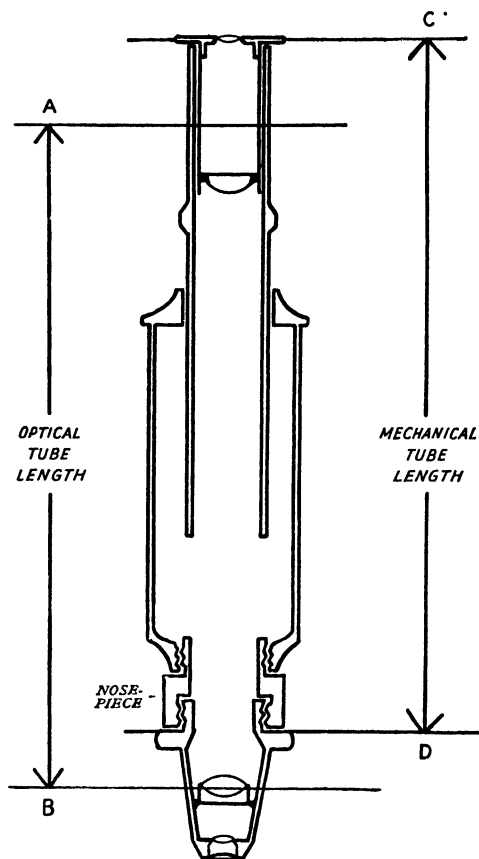
MAGNIFICATION

The objective works at a distance from the object somewhat less than its focal length. A real, inverted and enlarged image is formed in the upper part of the tube, and this real image is magnified further by the eye-piece. Thus, the total magnification is the product of the separate magnifications of the objective and of the eye-piece, and depends on three factors:—

- (1) The focal length of the objective.
- (2) The magnifying power of the eye-piece.
- (3) The distance between the lens system of the objective and the image produced—the “*optical tube length*.”

In actual practice however, when calculating the magnification, the “*mechanical tube length*” is used; the diagram on p. 45 illustrates optical and mechanical tube lengths.

The “*optical tube length*”—AB—is the distance between the posterior principal plane of the lens system of the objective



and the plane of the image which is in the upper part of the draw-tube.

The “*mechanical tube length*”—CD—is the distance between the eye-lens of the eye-piece and the point where the objective fits into the lower end of the body-tube or nose-piece. In adjusting the draw-tube to a given tube length, it must be

remembered that the draw-tube scale reads from the foot of the body-tube and does not include the nose-piece, the length of which is 18 mm. Allowance must be made for this—*e.g.* if the objective works at 160 mm. tube length, the scale of the draw-tube is set at 142 mm., which, with the length of the nose-piece, gives the required total length of 160 mm.

When calculating the magnifying power of a given objective and eye-piece, the optical tube length may be taken for practical purposes as equal to the mechanical tube length.

It is emphasized that objectives are designed to work at a definite tube length and any variation from this distance may seriously impair the definition obtained, particularly when apochromatic objectives are used.

The initial magnifying power of the objective is first determined, and then multiplied by the magnifying power of the eye-piece, when the total magnification is obtained. The objective acts as a convex lens and its magnification can easily be calculated as follows :—

$$\begin{aligned}\text{magnification of objective} &= \frac{\text{size of image}}{\text{size of object}} \\ &= \frac{\text{distance of image from objective}}{\text{distance of object from objective}}\end{aligned}$$

The distance of the image from the objective is the “optical tube length” which is approximately equal to the “mechanical tube length,” and this is determined from the draw-tube scale, adding 18 mm. thereto for the length of the nose-piece.

The distance between the object and the objective depends on the objective used, and this distance is adjusted by means of the coarse adjustment when the object is focussed. It may be taken as equal to the focal length of the objective. Suppose an object is examined with a 16 mm. ($\frac{3}{4}$ -in.) objective and a $10\times$ eye-piece, the tube length being 160 mm.; the size of the image produced by the objective alone depends on the ratio of the tube length to the focal length of the objective—*i.e.* 160 mm. : 16 mm. (ten times); this real image is now magnified ten times by the $10\times$ eye-piece, making a total magnification of 100 diameters. If a 4 mm. ($\frac{1}{4}$ -in.) objective is employed the distance of the image is the same (160 mm.), but the distance between objective and object is only 4 mm., hence the initial magnification of the objective is 160 : 4—*i.e.* 40. This is further magnified by the $10\times$ eye-piece to 400 diameters. Similarly a 2 mm. objective has an initial magnification of 80, and when used in conjunction with a $10\times$ eye-piece gives a total magnification of 800 diameters.

Thus : total magnification

$$= \frac{\text{tube length}}{\text{focal length of objective}} \times \text{eye-piece magnification.}$$

To find the magnification of any system of objective and eye-piece, divide the tube length employed by the focal length of the objective : multiply this figure by the magnification of the eye-piece and the total magnification is obtained.

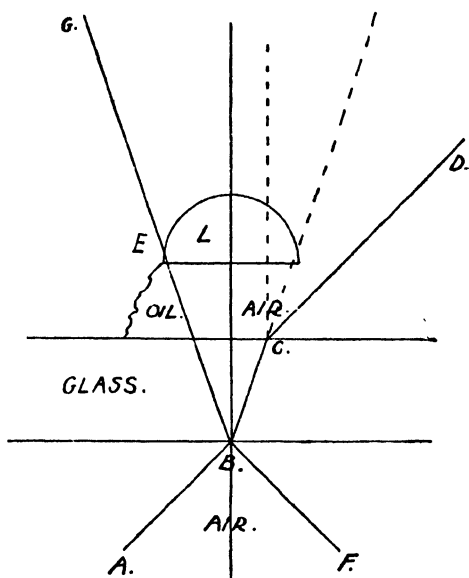


Diagram showing the paths of rays through (1) a dry lens (on right), and (2) an oil-immersion lens (on left) (after Spitta).

Note the refraction of the oblique ray *ABCD* in passing from the glass slide to air, as compared with the ray *FBEG*. *L* is the front lens of the objective.

It is thus seen that the magnification varies inversely as the focal length of the objective, the shorter the focal length the greater the magnification.

Makers now engrave the initial magnification of the objective on the objective mount, and as the eye-pieces are also designated by their magnifying power, the total magnification is easily and correctly determined, provided of course that the proper tube length is used.

The $\frac{1}{2}$ -in. objective has, in reality, a shorter focal length than that by which it is designated, and gives a magnification of 100 diameters. When used in conjunction with a $10\times$ eye-piece the total magnification is 1000 diameters.

The magnification usually employed in bacteriological work is 800–1000 diameters.

The $\frac{1}{2}$ -in. oil-immersion lens works very close to the cover-slip, and the intervening space between objective and cover-slip is filled with cedar-wood immersion oil. The reason for this is that when an oblique ray of light emerges from a dense medium (glass) into a rare medium (air) it is refracted outwards—*i.e.* away from the normal (see diagram, p. 47—ABCD). As the brightness of the image depends upon the light entering the objective, and the resolution (*vide infra*) depends on the effective aperture, this refraction of light diminishes not only the brightness but the clearness of the image. If, however, the space between objective and object is occupied by immersion oil, which has the same refractive index as glass, the rays of light do not undergo refraction and pass into the objective (see diagram—FBEG).

The high-power ($\frac{1}{6}$ -in.) is a “dry” lens and must *not* be used with immersion oil. Oil must be used only with lenses specially computed to work with this fluid. Such objectives have “oil immersion” engraved on them.

NUMERICAL APERTURE

Objectives are rated not only by their focal length but also by their *Numerical Aperture* (N.A.). The numerical aperture may be defined simply as the

ratio of the diameter of the lens to the focal length.¹ It is expressed mathematically as follows:—

$$\text{N.A.} = n \sin U$$

where n is the refractive index of the medium between object and objective (air, 1.0; cedar-wood immersion

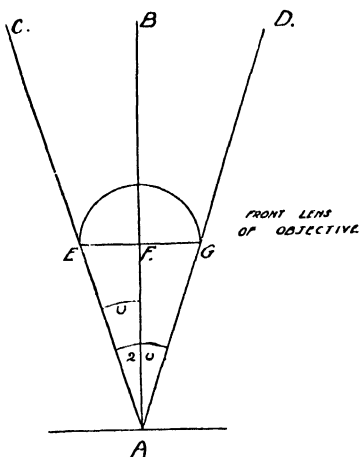


Diagram to illustrate numerical aperture.

oil, approximately 1.5), and $2U$ the *angle of aperture* — *i.e.* the angle formed by the two extreme rays of light, which, starting from the centre point of the object, reach the eye of the observer.

$$\begin{aligned} \text{That is, } \angle DAC &= 2U \\ \angle BAC &= U \\ \sin U &= \frac{EF}{EA} \end{aligned}$$

¹ The numerical aperture has been expressed in this manner to simplify description, but this is true only for objectives of long focal length, where EA is approximately equal to FA . With short-focus lenses of high numerical aperture this definition is not correct. The length EA , on which the numerical aperture really depends, is then very much greater than the distance of the objective from the slide (FA).

It is thus seen that the numerical aperture, other things being equal, depends on EF' , which is half the diameter of the lens. Lenses, therefore, may have equal focal lengths, but different numerical apertures depending on the diameter of the lens. It is to be noted, however, that the numerical aperture is not measured directly by the diameter of the lens, but is approximately the ratio of that diameter to the focal length.

The theoretical limit of the angle DAC is 180° —*i.e.* when the objective is actually on the object—and therefore the theoretical limit of U is 90° . The greatest possible N.A. of a dry lens cannot exceed 1, since the refractive index of air (n) = 1, and $\sin 90^\circ = 1$. Actually the highest practical N.A. of a dry lens is 0.95. On the other hand, the introduction of oil between the objective and object gives n a value of 1.5. The highest possible value, therefore, of $n \sin U$ for an oil-immersion objective is $1.5 \times \sin 90^\circ$ —*i.e.* 1.5. In practice, however, the highest N.A. of an oil-immersion objective (attained in an apochromat) is 1.40. The ordinary $\frac{1}{12}$ -in. objective for bacteriological purposes should have a N.A. of 1.30. The essential qualities of an objective depend on its numerical aperture, and these are :

- (1) *brightness of image*, which, other things being equal, varies as the *square* of the N.A. ;
- (2) *resolving power* and *defining power*, which vary directly as the N.A.

The *depth of focus*, while not dependent entirely on the N.A., varies in inverse proportion to it.

It is thus apparent why oil-immersion objectives give such good results—the N.A. being increased by the high refractive index of the oil. In general it may be said that in the case of two lenses of equal focal length the one with the higher N.A. is the better lens and is to be preferred. The 16 mm. or $\frac{2}{3}$ -in. objective should have a minimum N.A. of 0.28 ; the 4 mm. or $\frac{1}{4}$ -in. a minimum N.A. of 0.65 ; while the $\frac{1}{12}$ -in. oil-immersion should have a N.A. of 1.30.

The *resolving power* of a lens is its capacity to separate

two adjacent points (as apart from magnifying power). The limit of resolution is attained when the magnification reaches 1450 diameters, and on theoretical grounds objects smaller in diameter than half the wave-length of the light employed cannot be resolved. This theoretical limit cannot be obtained under visual working conditions, and in practice individual objects of less than 0.00025 mm. (0.25μ) in diameter cannot be resolved.

With ultra-violet light, however, which has a much shorter wave-length than visual light, greater resolution can be obtained.

Great advances with this method have been made by J. E. Barnard, using a specially constructed microscope, which requires special technical knowledge and skill for its use.

As optical glass does not transmit short-wave ultra-violet light, special quartz lenses have to be employed. The wave-length employed is so short that objects illuminated by it are not seen by the naked eye, but must be recorded photographically. By this means Barnard has succeeded in photographing several of the filterable viruses.

DEFINITION

This is the capacity of the objective to render the outline of an object distinct, and depends on the elimination of "spherical" and "chromatic" aberration.

Spherical aberration is caused by the periphery of the lens refracting more than the central portion. The peripheral rays, therefore, focus on the axis at a shorter distance from the lens than the central ones, with the result that the image is distorted.

Chromatic aberration is caused by the ray of white light being dispersed into its component colours as it is refracted through the lens, a spectrum being formed. The blue rays are refracted more, and come to a focus nearer the lens than the red rays. The different component colours do not come to the same focus and hence cannot blend to form white light. As a result, the image is fringed with colours and the outline is hazy.

Both chromatic and spherical aberration may be

corrected by the combination of lenses of different dispersive power—*e.g.* convergent convex lenses of crown glass having low dispersive power, and divergent concave lenses of flint glass having high dispersive power. By this means two of the spectrum colours are combined, and the ordinary achromatic objective is constructed in this manner.

APOCHROMATIC OBJECTIVES

While achromatic objectives fulfil all ordinary purposes, they are not sufficiently corrected for critical work, such as photography and resolution of minute objects, for which apochromatic objectives must be employed. These represent the highest degree of optical perfection, and are, in consequence, very expensive. Apochromats surpass all others in the matter of colour correction, and the essential factor in their construction is the use of the mineral *fluorite*. Fluorite possesses the following valuable optical properties:—

- (a) high degree of transparency;
- (b) low refractive index;
- (c) extremely small dispersion.

As a result of the use of fluorite at least three colours may be united, thus eliminating the secondary spectrum. This endows the objectives with a brilliance and “crispness” of image not attainable with ordinary lenses, and enables the maximum resolving power to be obtained.

Apochromatic objectives must be used only in conjunction with “compensating” eye-pieces, and care must be taken to adjust the tube length carefully and to employ a highly corrected and properly centered condenser.

A series of objectives containing a certain amount of fluorite, and which are intermediate in quality between the apochromatic and achromatic objectives, has been introduced by several makers. They are known as “semi-apochromatic” or “fluorite” lenses, and some of them have a performance approaching that of the apochromatic objectives. The oil-immersion

lenses of this series are very useful for dark-ground illumination.

The student or beginner in microscopy is advised not to purchase apochromatic or fluorite lenses, as the present-day achromatic or ordinary objectives have been brought to such a pitch of excellence that all routine examinations, and even research work, can be done with them.

CARE OF THE MICROSCOPE

The microscope is an instrument of precision, and care must be taken to preserve its accuracy. The instrument should be kept at a uniform temperature and not exposed to sunlight or any source of heat. When not in use it must be protected from dust under a cover or in its box. Failing these, it should be covered with a clean duster or cloth. The microscope should be cleaned at intervals and its working surfaces very lightly smeared with the finest machine oil. With binocular microscopes dust may collect on the surfaces of the prisms. This may be removed by passing a soft camel-hair brush down the eye-piece tubes after removing the eye-pieces. On no account must the prism case be opened and the prisms removed, as this will completely alter the optical alignment and necessitate the return of the instrument to the maker before it can be used again.

If the microscope has to be moved, it should be lifted by the upright limb, and not held by the body tube.

The oil-immersion objective must be cleaned each day after use by wiping the front lens with a well-washed silk or cotton handkerchief. There is on the market a fine tissue paper known as "lens paper," which is very suitable for the purpose. Oil remaining on the lens-front dries and becomes sticky; later it hardens and is then difficult to remove. Canada balsam accidentally present on the lens from a mounted microscopic specimen may also dry hard in the same way.

When cleaning the objective *do not use alcohol*, as the cement that unites the component lenses is soluble in alcohol, and in consequence the lens systems may become disorganised and the objective spoiled. Benzol or xylol must be used to remove dried oil, and if the oil is hard, repeated applications on a soft cloth are necessary.

Dry objectives—*e.g.* $\frac{3}{8}$ -in. and $\frac{1}{8}$ -in.—are cleaned with a piece of well-washed silk or fine cotton, or lens paper. If any oil or Canada balsam is accidentally present on the front lens it must be removed with a soft cloth moistened in benzol or xylol and the lens quickly dried with a soft cloth. On no account must the component parts of an objective be unscrewed.

DIRECTIONS FOR USING THE MICROSCOPE WITH CONDENSER AND OIL-IMMERSION LENS

Before commencing to examine a specimen special attention must be paid to the following:—

- (1) The objectives and eye-piece must be clean.
- (2) The draw-tube must be adjusted to the correct length.
- (3) The plane side of the mirror must be used.
- (4) The condenser must be properly fitted into the sub-stage, so that it can be racked up practically flush with the stage. In microscopes where the condenser is inserted from below into a sleeve fitting, the condenser is often not properly pushed into place and cannot be racked up sufficiently high for its focus to be in the same horizontal plane as the specimen.

*For bacteriological work it is recommended that artificial light always be used.*¹ A gas-filled electric lamp with a bulb of opal glass is highly suitable. It is convenient to

¹ We do not propose to discuss the relative advantages of daylight and artificial light. It is sufficient to point out that daylight is inconstant, and not always available, so that artificial light must then be employed. As the colours of certain preparations are different according to whether the illuminant is daylight or artificial light, the student is recommended to use a constant source of illumination—namely, artificial light.

have some form of microscope lamp which partially encloses the bulb, so that no glare reaches the eyes. It is not advisable to use the microscope at a window, as the daylight entering the eyes renders the vision less acute. A suitable arrangement is to use the microscope on a small table at one side of the room so that the observer's back is towards the window.

When examining an object, the manipulations of the microscope should be carried out in the following order :

- (1) Set up the microscope, place the object on the stage, and adjust the plane side of the mirror to the illuminant so that light is reflected into the condenser.
- (2) Focus the specimen with the low-power objective, using the coarse adjustment.
- (3) Manipulate the mirror until the image of the illuminant is seen in the centre of the field; lower the condenser so that the whole field is evenly illuminated.
(It is essential, particularly when examining tissues, to use the low-power first, in order to locate organisms and observe the tissue changes. A suitable field having been obtained, the slide must be kept in place by means of the right-hand clip if a mechanical stage is not used.)
Rack up the objective a short distance and place a drop of cedar-wood immersion oil on the portion of the specimen immediately below the objective.
- (5) Raise the condenser so that its upper surface is practically level with the stage, and make sure that the iris diaphragm is widely open.
- (6) Rotate the nose-piece until the oil-immersion lens is in position.
- (7) With the eye at the level of the stage lower the objective by means of the coarse adjustment until the lens is seen just to touch the oil.
- (8) Apply the eye to the microscope and observe

if the field is well illuminated. If not, adjust the mirror until maximum illumination is secured.

- (9) Carefully focus down, using the *coarse* adjustment, and when the object has come into view, use the fine adjustment to secure sharp definition. It is often necessary at this stage to raise or lower the condenser very slightly so that the optimum illumination is secured.

When the object is sharply focussed and the condenser is so adjusted that the image of the illuminant is seen in the field, the condition is termed *critical illumination*.

When using a binocular microscope the same directions should be observed, but, in addition, the eye-pieces should be adjusted to the correct interocular distance of the observer when the specimen is focussed with the low-power objective (*vide* direction No. 2).

When the observer has to examine a specimen for any length of time, as when searching for bacteria, he must adopt a comfortable position. Both forearms should rest on the table, and if there is no mechanical stage the slide is moved with the left hand while the right hand manipulates the fine adjustment.

EXAMINATION OF LIVING UNSTAINED ORGANISMS

“Hanging - drop” preparations are used for this purpose, and a glass slide having a circular concavity ground in the centre is employed.

There should be no difficulty in observing a satisfactory specimen if the following procedure is adopted:

- (1) By means of a match dipped in vaseline, a ring or square (according to the shape and size of the cover-glass) is outlined round the concavity.
- (2) With a platinum loop (*vide* p. 128) place a drop of fluid containing the organisms on a cover-glass laid on the bench.

- (3) Invert the slide over the cover-glass, allowing the glass to adhere to the vaseline, and quickly turn round the slide so that the cover-slip is uppermost. The drop should then be "hanging" from the cover-glass in the centre of the concavity.
- (4) Place the slide on the microscope, rack down the condenser slightly and partially close the iris diaphragm. (Excessive illumination renders the organisms invisible.)
- (5) With the low-power, focus the edge of the drop so that it appears across the centre of the field.
- (6) Turn the high-power ($\frac{1}{8}$ -in. or 4 mm.) lens into position and focus the edge of the drop. Obtain the best illumination by lowering or raising the condenser, and secure sharp definition by reducing the aperture of the diaphragm.

Instead of employing a hanging-drop preparation, a film of the fluid between an ordinary slide and cover-glass may be used.

Motility of organisms can be detected in this way. It is advisable to use the high-power dry lens and not the oil-immersion objective. Owing to the viscosity of the oil, the cover-slip is apt to move during focussing, and currents are thus caused in the fluid, which produce an appearance of motility in the organisms.

It is essential to distinguish between true motility, where the organism changes its position in the field, and Brownian movement, which is an oscillatory movement possessed by all small bodies (whether living or not) suspended in fluid (p. 8).

A *warm stage* is very convenient when examining fresh unstained preparations for amoebae and other protozoa. There are several types of warm stage on the market, some of which consist of a thin, flat metal box filled with hot water, or through which hot water can circulate, and having an aperture in the centre by which the light passes to the preparation. Other forms are electrically heated and have an automatic temperature control. The warm stage keeps the preparation at body temperature, and enables the movement of organisms

to be studied, as these movements may cease if the material is kept for any length of time at room temperature.

A simple warm stage may easily be improvised from a sheet of thin copper (18-gauge) shaped like the letter "T," with the long arm 5-6 ins. in length. The top of the "T" is the size of a microscope slide (3 in. $\times$ 1 in.) and in the centre is an aperture $\frac{1}{2}$ in. in diameter. The copper "T" is placed on the microscope stage with the long arm projecting forward, and the aperture over the condenser. The preparation is placed on the copper strip and secured by the stage clips. The projecting part of the "T" is warmed by means of a small Bunsen flame or spirit lamp. Care must be taken that the preparation is not overheated.

DARK-GROUND ILLUMINATION

This method renders visible delicate organisms, such as the spirochaete of syphilis, which cannot be seen in unstained preparations with an ordinary microscope.

By means of a special condenser the specimen is illuminated only by oblique light. The rays do not enter the tube of the microscope, and, in consequence, do not reach the eye of the observer unless they are "scattered" by objects (*e.g.* bacteria) of different refractive index from the medium in which they are suspended. As a result, the organisms appear brightly illuminated on a dark background.

Three requisites are necessary for adapting a microscope for dark-ground illumination :

- (1) A "dark-ground" condenser.
- (2) A suitable illuminant of sufficient intensity.
- (3) A stop which reduces the numerical aperture of the objective to less than 1.0, *if the ordinary oil-immersion lens is used.*

The Condenser.—A special condenser must be employed and may be of the paraboloid or of the concentric spherical reflecting type. The latter is recommended. The function of the special condenser is to focus the light on the object, the paths of the rays being such that no direct light passes into the front of the lens. The illustration shows the path of rays through the concentric reflecting condenser. The condenser

should be furnished with a centering device. It must be emphasised here that success with dark-ground illumination depends on the accurate centering of the condenser.

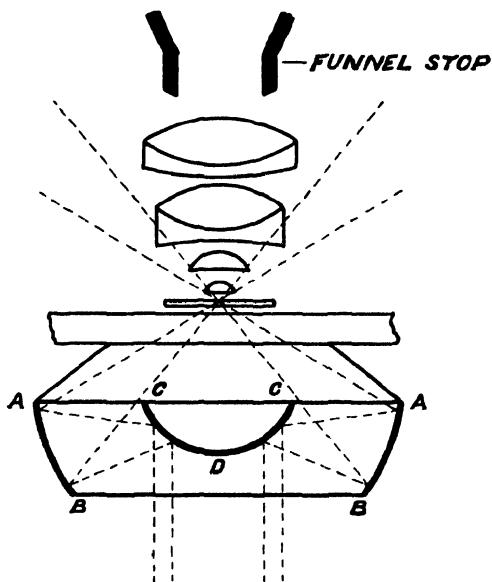


Diagram showing the paths of rays through the condenser and a $\frac{1}{8}$ -in. oil-immersion lens fitted with a funnel stop. AB and CDC are reflecting surfaces. The surface at CC is opaque. (After E. Leitz.)

The Illuminant.—A lamp of sufficiently powerful intensity should be employed. The small arc lamp, or the “pointolite” lamp, is well suited for the purpose. The arc lamp gives very good illumination and is supplied with a suitable condensing lens. The objection to it is that constant attention is required to keep the width of the arc adjusted as the carbons burn away. Arc lamps with a clockwork feed may be used to obviate this difficulty, but they are apt to

be unreliable. The pointolite lamp requires a special resistance, and the best results are secured with the direct-current model, although a type for alternating current may be obtained; in addition, an enclosed chamber with a condensing lens has also to be purchased. Its light is not so intense as that of the arc, but once adjusted this lamp requires no further attention, and gives a constant light for any length of time. On the whole, the pointolite is perhaps the more convenient illuminant when direct current is available.

In view of the fact that alternating current is becoming almost universal, the use of special small gas-filled lamps having a short, thick and tightly wound spiral filament should be noted. The source of illumination is small, compact and intense. Previous filament lamps had a long, loosely wound, thin filament, and a satisfactory beam of light could not be obtained. The newer lamps take 5 amperes at 6 volts, and can be worked from any alternating-current supply through a suitable transformer. As with the arc and pointolite lamps, a ventilated enclosed chamber with a condensing lens is necessary, and complete lamps at small cost can now be obtained.

The Funnel Stop.—When the objectives employed for dark-ground illumination have a numerical aperture of more than 1.0 (as in the case of ordinary oil-immersion lenses), a special stop to reduce the N.A. to less than 1.0 must be employed. This consists of a small funnel-shaped piece of vulcanite which fits into the objective behind the back lens. It is advisable to procure the stop from the maker of the lens employed. The stop is removed by unscrewing, and the objective can at once be converted for ordinary use.

Alternatively an objective adapter, with a small iris diaphragm, may be used. The front part of the oil-immersion objective is removed and screwed to the adapter, which then takes the place of the objective on the nose-piece. The numerical aperture of the objective may be reduced as desired by manipulating the iris diaphragm in the adapter.

Certain manufacturers have introduced for dark-ground illumination with bicentric condensers, special oil-immersion fluorite objectives, which are used without a funnel stop.

These are $\frac{1}{4}$ -in. N.A. 1.15, and $\frac{1}{8}$ -in. N.A. 0.95. The latter lens can be recommended for routine dark-ground observation, and in addition is very useful for histological work in place of the $\frac{1}{8}$ -in.

The Preparation.—The preparation should be as thin as possible in order to secure a satisfactory dark background, and so that the moving objects shall, as far as possible, be in one plane. A preparation which is too thick greatly diminishes the contrast in the dark field, and in order to obtain satisfactory contrast the objective has to be stopped down considerably, thus diminishing its resolving power. The preparation should not be too dense, otherwise there is an excessive number of particles which “scatter” the light. This causes lack of contrast even to a greater degree than a thick preparation. Some manufacturers supply special cells for dark-ground work so that when the cover-slip is placed on the slide the preparation has a definite and uniform thickness.

The thickness of the slide employed is important. The slides should be 1.0–1.1 mm. thick, and when a suitable supply has been obtained they should be used only for dark-ground work. They should be thoroughly clean and free from grease. The object to be examined must be at the focus of the condenser, the focal length of which is about 1.2 mm. If, therefore, too thick a slide is used, the focus of the condenser will be below the specimen and poor illumination will result; if the slide is too thin, the distance between the condenser and slide is such that a large amount of oil must be employed to make contact.

METHOD OF USING DARK-GROUND ILLUMINATION WITH THE OIL-IMMERSION OBJECTIVE

The microscope with special condenser and with the N.A. of the objective reduced by a funnel stop or iris-diaphragm adapter, is placed in front of the illuminant. It is advisable to have the microscope in the upright position and not inclined, to avoid

running of the oil. The condensing lens of the lamp is adjusted so that a slightly converging beam of light is obtained. With the plane side of the mirror the light is directed into the dark-ground condenser. Using the low-power ($\frac{2}{3}$ -in. or 16 mm.) objective, focus the surface of the condenser so that the engraved concentric rings on the surface come into view. These rings show the centre of the condenser, and if the condenser is out of centre adjust the centering screws so that the rings become concentric with the edge of the field.

When the condenser has no engraved rings the centering may be accomplished as follows:—

A slide preparation is placed on the stage and oil contact between it and the condenser established. The preparation is focussed with the $\frac{2}{3}$ -in. objective, and, particularly if an arc lamp be used and the mirror properly adjusted, a bright ring of light is noticed in the field. Focus the condenser up or down so that the ring of light contracts to the smallest bright spot obtainable. If this spot of light is not in the centre of the field the centering screws of the condenser must be altered accordingly.

The accurate centering of the condenser is of the utmost importance, and the time spent in this manipulation will be amply rewarded by the brilliant illumination obtained. The preparation to be examined must be covered with a No. 1 cover-slip, and it is advisable to ring round the cover-slip with vaseline to prevent evaporation. Place a large drop of immersion oil on the under surface of the slide and also on the top lens of the condenser, and a similar drop on the cover-glass. Place the slide on the microscope stage, taking care that the upper surface of the condenser is well below the slide. Rack up the condenser until oil-contact is made between the whole surface of the upper lens of the condenser and the slide; then bring the oil-immersion lens into position so that it touches the oil on the cover-slip. Adjust the mirror until the field, though dark, is evenly illuminated. Now carefully focus the specimen. A slight adjustment of the condenser, up or down, may be necessary, and some

manipulation of the mirror may also be required. After a little practice an evenly illuminated field with an intensely dark background and brilliantly lit objects may be obtained with a minimum of trouble.

Where much dark-ground examination has to be done, it is recommended that a microscope be reserved solely for this work, and kept ready with the illuminant in position, so that it is always available for immediate use. It is convenient to have the lamp and microscope fixed to a board for this purpose. The microscope, when not in use, should be covered to exclude dust.

After use the condenser and objective should be carefully wiped free from oil. Discarded preparations should be dropped into a covered jar of benzol kept for the purpose. When a sufficient quantity has been accumulated it will be found that they can easily be cleaned, as the oil and vaseline have been dissolved off by the benzol.

MICROMETRY

In bacteriological work the unit of measurement is 0·001 mm. designated a *micron* or μ . The measurement of microscopic objects is accomplished by means of the stage micrometer in conjunction with a micrometer eye-piece. The stage micrometer consists of a 3×1 in. slide on which is a millimetre scale graduated in hundredths of a millimetre. This scale is either engraved or made by a photographic process. The micrometer eye-piece consists of a special eye-piece in which a graduated scale, mounted on the diaphragm, can be focussed by means of the movable eye-lens.

When measurements are to be made the micrometer eye-piece is inserted into the draw-tube, the tube length being accurately noted, and the rulings on the stage micrometer focussed by the appropriate objective according to the size of the object to be measured. The number of divisions on the eye-piece scale corresponding to a definite number of divisions of the millimetre stage scale is determined. The stage micrometer is removed, and the object to be measured is next focussed. The number of divisions of the eye-piece scale which just cover the object are noted.

The millimetre value of each division of the eye-piece scale depends on the objective used, and the tube length employed, and is usually determined each time a measurement is taken.

Sometimes it is advisable to increase or diminish the draw-tube length so that the stage and eye-piece scales coincide or bear a geometric relation to each other—*e.g.* 1 division of the former to 10 of the latter.

For example, using a $\frac{1}{2}$ -in. objective and a $6 \times$ micrometer eye-piece at 165 mm. tube length, it was found that 100 divisions on the eye-piece scale exactly covered 11 divisions of the stage micrometer. Each division of the stage micrometer is $\frac{1}{100}$ mm.

100 eye-piece divisions = 11 stage divisions = .11 mm.

1 eye-piece division = .0011 mm. = 1.1μ .

1 eye-piece division, therefore, with the
given objective, eye-piece and tube
length = 1.1μ .

The stage micrometer was removed and a stained slide of blood showing malaria crescents was substituted. The diameter of a red blood corpuscle covered 7 divisions of the eye-piece scale—*i.e.* 7.7μ . A polymorph leucocyte covered 11 divisions, while the length of a malaria crescent was equal to 10 divisions, showing the sizes of these objects to be 12.1μ and 11μ respectively.

If the draw-tube is so adjusted that 1 division of the stage micrometer equals 10 of the eye-piece scale, then each division of the latter corresponds to 1μ .

Photographic Method of Micrometry.—A more accurate method is to photograph a film of the organisms or cells under a high magnification. Without disturbing the microscope or camera, the slide is removed from the microscope stage, and the stage micrometer substituted. A photograph of the stage micrometer is then taken at exactly the same magnification. By means of a pair of fine dividers the length of the organism on the print is taken, and its exact measurement found by applying this distance to the micrometer print.

CHAPTER IV

CULTIVATION OF MICRO-ORGANISMS

ONLY in exceptional cases can the identity of a bacterium be established by its morphological characters (p. 23). It is essential to obtain a *culture* by growing the organism in an artificial *medium*, and if more than one species or type are present these require to be carefully separated or isolated in *pure culture*. In this process there are three distinct operations:

- (a) The preparation of suitable culture media.
- (b) The removal of other organisms from the medium, glass-ware, etc., by sterilisation. As bacteria are ubiquitous, they are present in the material and on the articles used for making media. These contaminating organisms must be destroyed so that the culture medium is sterile.
- (c) The cultivation of the organism and its isolation from others present in the material to be examined. It is only occasionally that organisms can be grown directly from the body in pure culture.

CONTAINERS FOR MEDIUM AND CULTURES

In the past, flasks and test tubes, stoppered with cotton-wool, have been universally employed as containers for medium and cultures. Recently, however, improved methods for the distribution and storage of culture media have been introduced, in which screw-capped bottles of varying capacity and shape are used for these purposes and substituted for the original types of container. Thus, medium can be distributed and preserved in hermetically sealed bottles on the

same principle as the canning of foodstuffs. The designation "canned" has, for want of a better name, been applied to media bottled in this way. These methods are particularly valuable in large laboratories where media are prepared in quantity.

In the following pages the original methods are described, as in previous editions of the book, but the use of screw-capped bottles and "canned" media is specially dealt with for the guidance of laboratory workers, as apart from students who may be more interested in the ordinary methods applicable to the preparation and use of the simpler culture media.

PREPARATION OF GLASS-WARE

Washing and Cleansing.—New glass-ware requires special attention because of the resistant spores which are present in the straw and other packing material. Thorough mechanical cleansing with soap and hot water and the aid of a brush is not sufficient; neither are the spores always killed in the hot-air oven (*vide infra*). It is advisable to boil new glass for one hour in 5 per cent. soft-soap solution. Some of the soap powders on the market cause clouding of the glass and in consequence should not be used. Glass containers with discarded cultures are usually placed in 3 per cent. lysol immediately after use, but those containing spore-bearing pathogenic organisms, such as *B. anthracis* or *B. tetani*, must be autoclaved (p. 145). The discarded cultures and their containers are then boiled in 5 per cent. soft-soap solution for an hour in a covered boiler. The glass-ware is cleansed with a test-tube brush (or other suitable brush) and washed in running water to remove the soap. If the tap water is very "hard" and contains a considerable amount of calcium salts, rinsing in distilled water is necessary. The glass-ware is then allowed to drain and dry.

Neutralisation of Glass-Ware.—New glass-ware, especially the cheaper varieties, gives off free alkali, and this may be sufficient to interfere with the growth of certain organisms. Where such slight changes of reaction are of importance, the glass-ware should be soaked in 1 per cent. commercial hydrochloric acid for several hours, thereafter well washed in tap water, and finally rinsed in distilled water.

METHODS OF STERILISATION

Various methods are employed for the destruction of bacteria, according to the nature of the article

to be sterilised; and each method has its own particular use, with well-defined limitations. The usual methods of sterilising are either (1) destruction of the organisms by some form of heat or by chemical antiseptics, or (2) mechanical removal by filtration.

Chemical methods of sterilisation are not employed in the preparation of culture media, as the presence of the chemical which destroys the contaminating organisms will either kill or prevent the growth of the bacteria artificially introduced into the medium.

For the sterilisation of culture media and apparatus, heat is applied in some appropriate form, depending on the nature of the object to be sterilised.

STERILISATION BY HEAT

Objects may be sterilised by heat in two forms—dry heat (Bunsen flame or hot air) and moist heat (steam or hot water). Sterilisation by dry heat requires a much higher temperature, or a much longer time at the same temperature, than in the case of moist heat.

To ensure complete sterilisation, all forms of bacterial life must be destroyed, *and the time of sterilisation is that necessary to kill the most resistant forms*. Spores are extremely resistant to all methods of destruction, whether by means of heat or chemicals, and whereas the vegetative phase of an organism such as *B. anthracis* is killed by moist heat at 100° C. in a few seconds, the spores resist boiling for five minutes. Some spores may survive boiling for periods up to 1½ hours. The time for effective sterilisation, therefore, by any method is that which ensures complete destruction of all spores.

STERILISATION BY DRY HEAT

- (a) *Red Heat*.—Inoculating wires or needles, points of forceps, and searing spatulas are sterilised by this method, the heat from an ordinary Bunsen burner being utilised.

- (b) *Hot-Air Oven*.—In its original form this consists of a chamber having double walls between which hot air passes from a Bunsen burner. The oven may however be heated electrically. It is desirable to have an automatic device which keeps the temperature constant at any predetermined figure. A temperature of 160°C . for one hour or 180°C . for half-an-hour is necessary for complete destruction of bacterial spores. (It is obvious that such a temperature cannot be utilised for the sterilisation of culture media.) This is the best method for sterilising *dry* glass-ware, such as test-tubes, Petri dishes, flasks, throat swabs, quill tubes, graduated and capillary pipettes, and certain instruments, such as forceps and scissors and *all-glass* syringes. Test-tubes or flasks should be plugged with cotton-wool stoppers before sterilisation.

Formerly only thin glass-ware could be sterilised in this manner, as thicker glass cracked from uneven expansion. Since, however, the introduction of resistance glass, and strong flint glass for moulded machine-made bottles and other containers, articles of uneven thickness of glass, such as the screw-capped bottles referred to hereafter, can be sterilised repeatedly in the hot-air oven without risk of cracking.

Certain precautions have to be observed when sterilising glass-ware: (1) the glass should be perfectly dry; (2) the oven must be cold when the apparatus is inserted, then heated to the requisite temperature and kept at that temperature for the full time necessary for sterilisation; (3) the oven is allowed to cool before the articles are removed, as sudden or uneven cooling is also apt to cause cracking of the glass.

The advantage of this method is that all the articles are kept dry.

- (c) *Flaming*, by passing the article through the Bunsen flame without allowing it to become red hot. This method is useful for sterilising scalpels,

needles, the mouths of culture tubes, cotton-wool stoppers, glass slides and cover-slips. Needles and scalpels may also be sterilised by dipping them in methylated spirit and then burning off the spirit, the process being repeated several times.

STERILISATION BY MOIST HEAT

- (a) *Boiling in a Water-Bath*.—A suitable form of steriliser is the fish-kettle type made of enamel-ware or tinned copper. It should have a removable tray provided with a raised edge to prevent cylindrical instruments, such as syringes, from falling off. Five minutes at 100° C. is sufficient to kill all non-sporing organisms; certain spores, however, may resist longer exposures, even up to $1\frac{1}{2}$ hours. This method of sterilisation has only a limited use in bacteriology, and is employed for tubes, instruments (forceps, scissors, etc.), syringes, pipettes, measuring cylinders, etc. The addition of a small amount of sodium carbonate obviates rusting of steel instruments, but with stainless steel this is not necessary.

The interior of a test-tube may be sterilised quickly for ordinary purposes by boiling water in it.

- (b) *Steam at 100° C.*—This method is extensively used in bacteriology. A Koch or Arnold steam steriliser heated by steam, gas, or electricity with an automatic regulator is employed, and is particularly useful for the sterilisation of culture media. Its advantages are: (1) the apparatus need not be costly; (2) both the container and the medium are sterilised; (3) as the medium is in an atmosphere of steam, there is no loss from evaporation; (4) the apparatus requires little or no attention.

Sterilisation is effected in two ways:

- (1) One exposure for $1\frac{1}{2}$ hours. This usually

ensures complete sterilisation, and can be used for such media as broth or nutrient agar. It cannot be used for nutrient gelatin, as this medium, after prolonged heating, fails to solidify on cooling.

(2) Exposure at 100° C. for twenty minutes on each of three successive days. The accepted principle of this "intermittent" method of sterilisation is that one exposure is sufficient to kill the vegetative forms of bacteria; between the heatings, the spores, being in a favourable medium, become vegetative forms which are destroyed during the subsequent heating. This method is very useful for sterilising media containing sugars which may be decomposed by higher temperatures or by prolonged heating. It is employed also for the sterilisation of gelatin media.

It is advisable to cover the cotton-wool stoppers of tubes or flasks with two or three layers of parchment paper or "kraft" brown paper to avoid drenching.

- (c) *Steam at High Pressure in the Autoclave.*—The principle on which the autoclave is used is that water boils when its vapour pressure is equal to the pressure of the surrounding atmosphere. If, therefore, the pressure be increased inside a closed vessel, the temperature at which the water boils will rise above 100° C., the exact temperature depending on the pressure employed. The autoclave consists of a cylinder of gun-metal which is supported by a sheet-iron case. The lid is fastened down by screw clamps, and is rendered air-tight by means of an asbestos washer. The apparatus is furnished with a steam-tap and pressure-gauge, and also a safety-valve, which can be set to "blow off" at any desired pressure. The pressure originally employed was 15 lbs. per square inch above atmospheric pressure, making a total of 30 lbs.

per square inch absolute pressure. With this pressure water boils at $120^{\circ}\text{C}.$, and fifteen minutes' exposure to this temperature kills all forms of organisms, including the most resistant spores. This pressure is still used for sterilising empty glass containers, pathogenic cultures containing spores, dressings, etc. When sterilising agar and broth, the high temperature resulting from 15 lbs. of steam pressure may have a detrimental effect on the nutritive properties of the medium. An exposure of thirty minutes at 5 lbs. pressure is preferable. Autoclaving must not be used for sugar media or gelatin, as the former are decomposed and the latter will not solidify on cooling.

Directions for using the Autoclave.—See that there is sufficient water in the bottom of the cylinder. Insert material to be sterilised and light the gas. Place the lid in position, see that the tap is *open*, adjust the safety-valve to the required pressure¹ and screw down the lid. As the autoclave heats, air is forced out of the tap and eventually steam issues. *Make sure that all air has been expelled from the cylinder, as indicated by steam issuing freely*, and then close the tap. The pressure now rises until it reaches the desired level, when the safety-valve opens and the excess steam escapes. Allow the sterilisation to continue for the requisite time, fifteen or thirty minutes as the case may be, *from this point*. When sterilisation is complete, turn out the gas and allow the autoclave to cool until the pressure-gauge indicates that the inside is at atmospheric pressure. Now open the tap and remove the lid. If the pressure is suddenly released, the liquid media, being at a temperature above $100^{\circ}\text{C}.$ and suddenly exposed to ordinary atmospheric pressure, will

¹ In some varieties of autoclaves the adjustment of the safety-valve has to be determined previously by trial.

boil violently and be expelled from their containers with almost explosive force. In order to avoid drenching by the steam, cotton-wool stoppers should be covered with parchment paper or kraft brown paper.

Autoclaves heated by other means—*e.g.* steam or electricity—are operated in exactly the same way.

STERILISATION AT LOW TEMPERATURES

The sterilisation of serum or body fluids containing coagulable protein can sometimes be effected by heating for one hour at 57° C. on several successive days. The principle is the same as in the case of intermittent sterilisation at 100° C. (*vide supra*). It may be necessary to repeat the heating eight times to ensure complete sterilisation. Care must be taken not to allow the temperature to rise above 59° C., as inspissation may occur. The exposure to 57° C. is best carried out in a water-bath, but a 57° C. oven may be used. This procedure is not always effective—*e.g.* if certain resistant types of sporing organisms are present.

Vaccines.—Vaccines should be sterilised in a water-bath at a comparatively low temperature, one hour at 60° C. being *usually* sufficient. Higher temperatures may diminish the immunising power of the vaccine.

OTHER METHODS OF STERILISATION EMPLOYED FOR SPECIAL PURPOSES

STERILISATION BY CHEMICALS

- (a) Volatile antiseptics—*e.g.* chloroform. This method is used often in the sterilisation and preservation of serum (for culture media) and the chloroform, which is added in the proportion of 0.25 per cent., can later be removed by heating at 57° C. Chloroform is used also for preserving media in bulk.
- (b) Antiseptics of the phenol group. Liquor cresolis saponatus (lysol) and cresol are powerful antiseptics. Their chief use in a laboratory

is for sterilising surgical instruments and discarded cultures, and for disinfecting the hands and killing cultures accidentally spilt by the worker. Lysol is generally used as a 3 per cent. solution. Carbolic acid, 0·5 per cent., or tri-cresol, 0·3 per cent., is used for preserving sera and vaccines.

- (c) **Metallic salts.** One of the most important in this group is perchloride of mercury (1 : 1000 solution). A bowl of this solution (conveniently prepared from tablets) should be kept on the laboratory bench for sterilising the hands after working with infected material or cultures.
- (d) **Glycerol.** This is used as a 50 per cent. solution for the preservation of certain of the viruses, which retain their virulence in it for many months. In addition the glycerol kills off contaminating organisms, so that in time the virus-material contains no living bacteria of the ordinary type. This method is used in the preparation of vaccinia virus for vaccination against smallpox (*vide* p. 472). Glycerol is also used as a preservative of agglutinating and other sera (*vide* p. 203).

STERILISATION BY FILTRATION

This is effected by the use of filters of unglazed porcelain (Chamberland, Maassen type) or of diatomaceous earth (Berkefeld, Mandler clay filter), the pores of which are so small that ordinary bacteria are prevented from passing through.

Seitz filters, in which filtration is effected through asbestos "films," are very useful and reliable. The film consists of a flat disc of asbestos material of special composition, and is inserted into special metal holders which ensure a tight joint being made. After use the asbestos film is discarded and a new one employed for each filtration. The films are inexpensive, and filters of various sizes may be obtained according to the amount of fluid to be handled. The

large size of Seitz filter, with 14 cm. diameter films, can be recommended for the sterilisation of large amounts of serum to be used in the preparation of media (*vide p.* 103).

Extremely fine filters made of collodion membranes are used in the study of the filterable viruses.

Further details are given on pp. 457, 483. ✓

MAINTENANCE OF STERILITY

It is necessary that apparatus, after sterilisation, should be kept sterile.

Test-Tubes and Flasks. — The interiors of test-tubes, flasks, bottles, etc., must be carefully protected from bacterial contamination due to access of air, dust, etc., before and after the addition of medium and during the subsequent cultivation of organisms. This is usually done by means of cotton-wool stoppers. These should be $1\frac{1}{4}$ – $1\frac{1}{2}$ in. long, $\frac{3}{4}$ –1 in. inserted into the mouth of the tube, etc., and the remainder projecting. They should fit firmly, but not so tightly as to render their removal difficult.

Stoppering of Tubes. — Long fibre cotton-wool is essential, and must be free from short broken fibres and dust. Non-absorbent cotton is preferable, because, after steaming, plugs tend to remain moist, and if the medium is to be kept for any length of time, and absorbent wool is used, moulds will grow through the stopper and contaminate the medium. A sufficient amount of cotton-wool (*vide supra*) should be forced into the tube with a rod or pair of forceps, and the cotton-wool should not be twisted in, as creases are formed along the sides of the glass which act as channels for contaminating organisms.

Instead of the ordinary roll of cotton-wool being used, it is recommended that the non-absorbent wool be obtained in the form of a long thin ribbon known as “rope wool” or “neck wool” of the type used by hairdressers. It is kept in a tin container with a hole in the lid, and the appropriate amount of wool for the stopper is easily obtained without waste.

When tubes or flasks have to be stored for any length of time the stoppers should be covered with sterile kraft paper, kept in place by means of fine string or a rubber band. Sterile rubber stoppers may, in some

cases, be used instead of cotton-wool, particularly where the contents of the flask or tube have to be kept a considerable time, as in the case of immune sera, or where these vessels have to be transported by post or by messengers ; see also blood culture, p. 141.

Screw-capped Bottles.—Screw-capped bottles of 6, 12 and 20 oz. capacity (*vide* p. 79) can be used for storing media instead of flasks, and small 1 oz. screw-capped bottles (*vide* p. 126) instead of test-tubes. Their use is referred to later.

Petri Dishes.—Each individual dish should be wrapped in kraft brown paper before sterilisation, and kept in the paper until used. For a 4 in. dish the size of paper should be 12 in. square. They may also be sterilised and kept in cylindrical copper boxes.

Pipettes.—1 c.c. and 10 c.c. graduated pipettes should be wrapped in a long strip of kraft paper, which is wound round them in a spiral manner before sterilising in the hot-air oven. Bulb pipettes (10 c.c., 50 c.c. etc.) are also covered with kraft paper. Under these conditions pipettes remain sterile in their wrappers for considerable periods of time.

Capillary pipettes are sterilised in large test-tubes, 15 in. $\times$ 2½ in., having a cotton-wool stopper, or in copper boxes. The former method is preferable.

Ampoules are sterilised in the hot-air oven with the necks sealed and are kept in metal boxes.

PREPARATION OF CULTURE MEDIA

The majority of the organisms to be studied are pathogenic, and in order to obtain suitable growths the artificial culture media should approximate to the composition and reaction of the tissues and body fluids in which these organisms grow.

FOOD SUPPLY AND ENERGY REQUIREMENTS OF BACTERIA

Bacteria derive their energy by oxidations and by the decomposition of organic food material such as

proteins and carbohydrates. At the same time simple organic substances—*e.g.* amino-acids and other products of protein disintegration—are utilised in the synthetic metabolism of these organisms.

The food requirements of bacteria vary with the natural environment and the particular rôle they have in nature, but generally speaking the pathogenic organisms require a medium approaching in composition and reaction to the body tissues and fluids. Some organisms are able to grow under a wide range of conditions, whereas other more highly parasitic bacteria, such as the gonococcus, are restricted in their requirements, with regard not only to food but also to temperature and other factors. It is usually impossible to reproduce exactly the natural conditions under which pathogenic micro-organisms live. On the other hand it must be realised that a considerable degree of adaptability exists among them, and for the great majority of pathogenic bacteria suitable artificial media have been found.

All bacteria require nitrogen, and in the case of the pathogenic organisms it is generally obtained from protein. In the usual culture media nitrogen is conveniently supplied in the form of "peptone" which is a commercial product obtained by peptic digestion. Peptone is a crude product and consists of a mixture of proteose, peptone, polypeptides and amino-acids. This simpler digested form of protein is used because it is soluble and does not coagulate on heating. Moreover it is better adapted to synthetic metabolism than native protein, especially if it contains a sufficiency of amino-acids. Alternatively, instead of adding the digested protein in the form of commercial peptone, complex native proteins are split up during the preparation of the medium by the action of pepsin or trypsin to form simpler uncoagulable nitrogenous compounds. This is the principle of the so-called "digest media," of which Hartley's broth (*vide* p. 83) is an example. Other forms in which protein is provided are serum protein—*e.g.* serum-agar and Löffler's

medium—and egg protein as exemplified by Dorset's egg medium for the tubercle bacillus.

Carbon is also a constituent of bacterial protoplasm and is derived mainly by the breaking up of carbohydrates. This process is essential in the case of some organisms, and certain bacteria can grow on an almost pure carbohydrate medium such as potato. Carbohydrates are supplied generally in the form of sugars (*vide* p. 100), and they are usually valuable accessories to growth, especially if fermented—e.g. *B. diphtheriae* grows much better if a small quantity of glucose be added.

Vitamins are important factors in the growth of certain pathogenic bacteria, and these are supplied in the form of *fresh* body fluids, such as blood, serum and ascitic fluid, which at the same time serve as a rich supply of protein. As an example of vitamin requirements, the growth of *B. influenzae* (*vide infra*) may be taken. It must be recognised that vitamins are susceptible to heat and adsorption, and that excessive heating and filtration of media must be avoided if these accessory growth factors are to be retained (*vide* "hormone" agar, p. 88).

Mineral salts are essential to growth, particularly the chlorides, phosphates and sulphates among the acid radicles, and calcium, potassium and sodium among the bases.

The value of meat extract in culture media is recognised although its precise properties are doubtful. In composition it is practically devoid of protein, and consists of mineral salts, a small amount of sugar and some nitrogenous muscle extractives.

While some pathogenic organisms multiply on a comparatively simple medium, it is usually necessary to supply protein, carbohydrates and mineral salts if a profuse growth is required.

There are certain pathogens with particular requirements which have to be fulfilled before growth takes place. Some highly parasitic organisms will not grow without the presence of serum—e.g. the gonococcus.

The haemophilic bacteria—e.g. *B. influenzae*—do not grow on ordinary media, but multiply if blood is present; two growth-promoting factors present in blood designated “X” and “V” appear to be essential. The “X” factor is thermostable and resists autoclaving at 120° C. It acts like a peroxidase and is associated with the haemoglobin. It apparently enables the organism to assimilate atmospheric oxygen. The “V” factor is more easily destroyed by heat, and is of vitamin nature. Egg media are especially suitable for the growth of the tubercle bacillus. The bacillus of John’s disease can be grown if an extract of other acid-fast bacilli is present. Generally speaking the filterable viruses do not multiply on the usual artificial media, but certain of them have been cultivated in association with tissue cultures—e.g. vaccinia virus.

Certain atmospheric conditions also are essential for the growth of various bacteria; these are dealt with on pp. 15, 136.

“CANNED” MEDIUM

Hitherto culture media have been stored in bulk in flasks with cotton-wool or rubber stoppers, or in some form of “milk” or “whisky” bottle. Recently there has been introduced the “canned” medium principle.¹ This consists essentially in placing the medium in a container, which is then hermetically sealed, and thereafter sterilised. The result is that, the container being completely closed, the contents remain sterile indefinitely, in exactly the same way as canned foods. There is no necessity to store in a cold room, while transport is much more easily accomplished. The principle is applied not only to media in bulk, but also in smaller quantities, and small screw-capped bottles can now completely replace the test-tube for bacteriological purposes. It should be noted that though the “canned”

¹ For further details and arguments see J. E. McCartney, “Screw-capped Bottles in the Preparation and Storage of Culture Media,” *Lancet*, 1933, ii. (19th August), p. 433.

medium principle was introduced to facilitate the distribution, storage and transport of culture media in a large laboratory organisation, it can equally well be applied in smaller laboratories, and where comparatively small quantities of culture media are made and used. It is particularly recommended that the methods of using screw-capped bottles be adopted, as a considerable saving in material and labour will result.

The orthodox methods at present employed are given throughout the succeeding pages, but the application of the "canned" medium principle is also indicated.

For culture media in bulk, screw-capped plain medical bottles¹ are employed. They are supplied in various sizes, ranging from 1 oz. to 20 oz., but the sizes most commonly used are 4 oz., 6 oz., 12 oz. and 20 oz., in which are placed 50, 100, 250 and 500 c.c. of media respectively. The bottles are of the same shape as ordinary medicine bottles ("medical flats"), but have no graduations or markings on them. They are of clear white flint glass and the neck has an external screw thread. The screw caps are made of aluminium sprayed with white cellulose paint, and the washer is made of waxed millboard. The bottles are received from the manufacturers already cleaned, washed, capped and sterilised, so that they require no further treatment before use. This is a great advantage, as all the elaborate cleaning of unused glass-ware as described on p. 66 is obviated. As a substitute for test-tubes small round screw-capped bottles of 1 oz. (28 c.c.) capacity (see Figure on p. 126) are used. In order to prevent the washer from adhering to the lip of the bottle, instead of remaining inside the cap, a disc of black enamelled paper known as "blackol" may be placed as a liner over the millboard washer.

The "medical flats" can be autoclaved with the cap tightly screwed on, either empty or containing media, with little risk of breakage. Care must be taken that the bottles are placed in the steriliser

¹ Made by United Glass Bottle Manufacturers Ltd., 40-43 Norfolk Street, Strand, London, W.C.2.

loosely, and not packed tight in a wire crate or other container, otherwise breakages will inevitably occur.

IDENTIFICATION OF MEDIA

Coloured beads are used in order to identify the various bottled media, and to avoid the use of gummed labels, which may become detached in the steamer when a solid medium is melted. These are the ordinary opaque beads for threading necklaces, 7 mm. in diameter, and are inexpensive to buy. (The clear glass beads are not suitable.) They are first treated in a 1 per cent. solution of commercial hydrochloric acid, well washed with tap water, then distilled water, and finally dried in the oven. The appropriate bead is dropped into the bottle before filling. Owing to the curve at the bottom of the bottle, the bead remains in one corner, and is very easily recognised no matter what type of culture medium is used. On tilting the bottle for pouring, the bead comes to rest on the shoulder and remains in the bottle, even when the bottle is almost completely inverted. The coloured beads can be used for identification of all kinds of media and reagents. The colours employed are as tabulated, and these are recommended as a standard series.

Identification Colours

<i>Culture Medium</i>	<i>Colour of Bead</i>
Digest broth	Black
Nutrient agar made from digest broth	Black
Infusion broth	Yellow
Nutrient agar made from infusion broth	Yellow
MacConkey's medium (see note on p. 112)	Red
Peptone water without indicator	White
Peptone water with indicator	Brown
Glucose media	Green

Solutions, etc.

Distilled water	White
Normal saline	Dark blue
Carbol-saline	Light blue

BROTH (or BOUILLON), NUTRIENT GELATIN AND AGAR

The basis for the media ordinarily employed in the study of the common pathogenic bacteria is nutrient broth. Agar or gelatin is frequently added merely to solidify it.

It should be noted here that the various "digest" media to be described are of great value for obtaining very luxuriant growths of organisms, but the cultures tend to die out rapidly. For maintaining stock cultures the use of media prepared from ordinary meat extract is advisable.

BROTH

The first stage in the preparation is the making of a watery extract of meat. The type of meat used is an important factor in the quality of the broth obtained. Fresh lean beef or ox heart is quite satisfactory. Horse-flesh is cheaper, but is usually not so fresh, and, in addition, contains a higher percentage of fermentable sugar, which may make the broth unsuitable for many purposes, such as the preparation of toxins (*vide* p. 284). The meat is carefully freed from fat, minced as finely as possible, and added to tap water in the proportion of 500 grams to 1 litre. After extraction for twenty-four hours at a low temperature—*e.g.* in the refrigerator—the mixture is strained through muslin to keep back the small particles of meat, and the meat residue expressed in a meat-press. The fluid is bright red in colour and there is often a thin layer of fat on the surface, which may be removed by skimming with a piece of filter paper. It is boiled for fifteen minutes, or steamed in a steam steriliser for two hours, when it becomes brown in colour and turbid on account of the alteration of the haemoglobin and the small particles of coagulated protein. It is now filtered and the clear fluid is made up to the original volume by the addition of distilled water. The unheated extract contains soluble proteins, salts, extractives and haemoglobin. As a result of the heating the meat proteins are

coagulated, and are removed by filtration. The finished extract should be clear and light yellow in colour, but is not suitable as a culture medium owing to the lack of nitrogenous material. Digested and uncoagulable protein in the form of commercial peptone¹ is added in the proportion of 1-2 per cent., and the salt content is increased by the addition of sodium chloride (0·5 per cent.). These are dissolved by heat and the extract is again filtered. Owing to the sarcolactic acid present in the meat, the reaction of the extract is acid, and this reaction is unsuitable for the growth of most organisms. When the reaction has been adjusted to the optimum by suitable means (*vide* p. 89), the medium is sterilised in the autoclave or steam steriliser, and the resulting preparation is designated Infusion Broth or Bouillon.

An alternative method employed by us for making broth gives very good results and is quicker to carry out.

Meat (freed from fat and minced)	500 grams
Tap water	1000 c.c.

Place the mixture in the steriliser and steam for three hours. Filter and make up to the original volume with water. This constitutes the "meat extract."

The broth is made from this extract as follows :

Meat extract	1000 c.c.
Peptone (1 per cent.)	10 grams.
Sodium chloride (0·5 per cent.)	5 grams.

The mixture is placed in the steamer for fifteen minutes to allow the ingredients to dissolve. Add normal caustic soda till the reaction is faintly pink to phenol phthalein, then place in the steamer for forty-five minutes to precipitate phosphates. Filter while hot and make up to the original volume.

The reaction is adjusted to the required pH (*vide* p. 91) by the usual method, except that the medium is alkaline and must be titrated with N/20 HCl, and acid added to give a reaction of pH 7·5.

It is emphasised that the minimum amount of heating of media is desirable, as overheating damages the nutritive properties.

Infusion broth and nutrient agar made from it are identified by means of a YELLOW bead in the container (*vide* p. 80).

¹ For ordinary purposes "commercial" peptone serves admirably, but for special purposes sugar and indole must be absent.

Lab.-Lemco.—There is on the market a meat extract known as “Lab.-Lemco,” which may be used as a substitute for the extract of fresh meat in the proportion of 10 grams of Lab.-Lemco to a litre of water. The addition of salt (0·5 per cent.) and peptone (1 per cent.) converts it into ordinary broth. The reaction must be standardised as in the case of the ordinary meat-extract broth.

DIGEST MEDIA

In the ordinary media, digested protein is added in the form of peptone, but in the “digest” media the meat is digested by the action of trypsin, and the products of this digestion constitute the basis of the medium. The simpler uncoagulable nitrogenous compounds necessary for the growth of organisms are therefore not added as such, but are formed during the preparation of the medium.

A useful form of “digest” medium for general use, which is also particularly suitable for the production of diphtheria toxin, is :

HARTLEY'S BROTH

Ox heart or lean beef (free from fat and minced)	1500 grams
Tap water	2500 c.c.
Mix together and heat in the steam steriliser until a temperature of 80° C. is reached. Then add :	
Sodium carbonate (anhydrous) 0·8 per cent. solution (cold)	2500 c.c.
Cool to 45° C. and add :	
Pancreatic extract ¹	50 c.c.
Chloroform	50 c.c.

¹COLE AND ONSLOW'S PANCREATIC EXTRACT

Fresh pig pancreas (fat-free and minced)	500 grams
Distilled water	1500 c.c.
Absolute alcohol	500 c.c.

Shake the mixture thoroughly in a large stoppered bottle and allow to stand for three days at room temperature, the shaking being repeated occasionally. Strain through muslin, and filter through Chardin paper. Measure the filtrate and add pure strong hydrochloric acid in the proportion of 0·1 per cent. This causes a cloudy precipitate which settles in a few days and can be filtered off.

Filtration of the extract is not essential and it can be kept exactly as mixed, with the addition of hydrochloric acid.

This extract keeps indefinitely if stoppered bottles are used. If used at once the hydrochloric acid need not be added as its use is to retard the slow deterioration of the trypsin.

The mixture is then incubated at 37° C. for six hours, the liquid being frequently stirred. After digestion is completed, add 40 c.c. of pure strong hydrochloric acid, steam for half-an-hour, and then filter.

If the broth is to be stored in bulk, adjust the reaction with normal solution of caustic soda so as to be neutral to phenol phthalein. Bottle, and steam for twenty minutes. When cool add 0·25 per cent. of chloroform and store in the cold-room. When required for use steam for twenty minutes to remove chloroform, filter and adjust to the required reaction. The broth is made alkaline for storage purposes.

It is very convenient, however, to store the broth in 250 c.c. and 500 c.c. amounts in 12 and 20 oz. screw-capped medical flats (p. 79), and also to put up the broth, immediately after being made, in 100 c.c. amounts in blood-culture bottles (p. 142). After making the broth neutral to phenol phthalein, it is steamed for one hour to precipitate phosphates and filtered whilst hot. When cool, the reaction is adjusted to pH 7·6. The broth is then bottled, the caps tightly screwed on and the whole batch sterilised.

Digest broth and the nutrient agar made from it are identified by a BLACK bead in the container.

Broth being a fluid medium has certain disadvantages:

- (1) growths do not exhibit specially characteristic appearances in this medium, and therefore it is of little use in identifying species;
- (2) organisms cannot be separated from mixtures by growth in this medium (*vide* p. 132).

It can, however, be rendered solid by the addition of gelatin, 10–15 per cent., or agar-agar, 2 per cent.

NUTRIENT GELATIN

This medium, first introduced by Koch, is made by dissolving the best sheet-gelatin, 10–15 per cent. by weight, in broth. Solution is effected by heating in a steam steriliser. The mixture is filtered and cleared with white of egg (see Agar). The finished product is sterilised in the steam steriliser—twenty minutes each day on three successive days. Prolonged exposure to 100° C. or autoclaving destroys its property of solidifying when cooled, thus rendering it useless for bacteriological purposes.

The resulting medium is perfectly transparent when solid, and should be of firm consistence, yet not so stiff that it is split by the needle when inoculated (*vide* p. 125).

The proportion of gelatin used varies with the time of year, and in very hot summer weather 15 per cent. may be necessary. A suitable consistence may be obtained by adding 10 per cent. in the winter and 12 per cent. in the summer. Gelatin at this strength melts at about 24° C., and is therefore fluid at incubator temperature. On this account it has a restricted use, and is employed only for organisms which grow at room temperature.

Gelatin is an albuminoid, and therefore is digested and liquefied by the proteolytic ferments of many bacteria. This property of liquefying gelatin is used as a means of differentiating certain organisms. As gelatin is not frequently used in routine work it should be kept in small screw-capped bottles (*vide* p. 126). When the medium is made it is distributed into the bottles, the cap tightly screwed on, and the whole batch sterilised. Under these circumstances the medium keeps indefinitely. After the gelatin has been inoculated (*vide* p. 125) the cap is again tightly screwed on, which prevents evaporation during the growth of the organism.

NUTRIENT AGAR

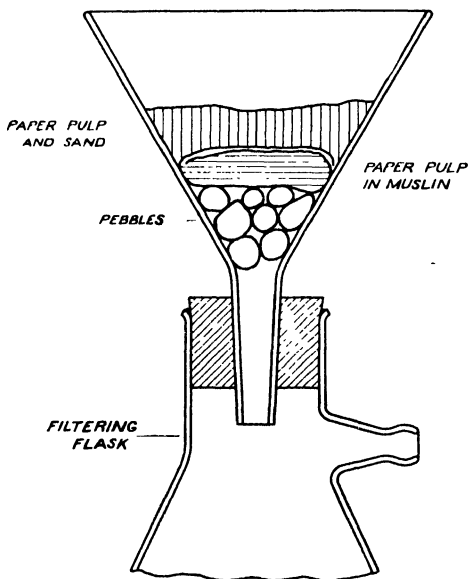
Agar-agar, or "Agar" for short, comes into the market in the form of dried strands of a seaweed found in the Chinese seas. It is also obtained as a powder, which is preferable.

Agar powder or fibre as bought contains many solid impurities, and when nutrient agar is made it must be filtered to produce a clear and transparent medium. Owing to the large amount of minute colloidal particles in solution, filtration through filter paper is slow, and in consequence before filtration the nutrient agar is "cleared" with egg white or egg albumin.

The proportion of agar generally used to solidify broth is 2 per cent. of the powder, and 2·5 per cent. of

the fibre. After the agar has been added, the broth is placed in the steam steriliser for one hour to effect solution. The mixture is then cooled to 55°C. , and the switched whites of two eggs, or 10 grams of egg albumin dissolved in 50 c.c. water, are added per litre. The medium is now steamed for two hours, or preferably autoclaved for one hour at a pressure of 5 lbs. per square inch. The agar solution is then filtered through Chardin filter paper in the steamer, distributed into bottles or tubes, and finally sterilised either by autoclaving at 5 lbs. pressure for half-an-hour, or by steaming for half-an-hour each day on three successive days.

When dealing with large quantities of medium, it will be found quicker and easier to filter nutrient agar through sand



and paper pulp instead of through filter paper. For amounts up to five litres we can recommend the following method. Into a 10 in. glass funnel place about a dozen round clean

pebbles to form a support for the sand and paper pulp. The paper pulp is thoroughly shredded and soaked in warm water. Take a large sheet of muslin 24 in. square, and place it over the funnel with the ends hanging over the side. Place moist paper pulp on top of the muslin until the layer is two inches deep, and pack it firmly (with the bottom of a 100 c.c. cylindrical measure), especially round the edge. Turn the ends of the muslin over the surface of the paper pulp, so as to form a sort of bag. Now superimpose on this a one-inch layer consisting of equal parts of moist paper pulp and well-washed coarse, sharp, builder's sand. The funnel and its contents are placed in the steamer or autoclave at the same time as the broth and agar powder when the latter is to be dissolved. It is important to note that no egg or egg albumin is required. The funnel is taken while hot from the steriliser, connected to the water-pump or other suction apparatus (*vide* p. 483), and melted agar poured into it. Gentle suction is applied and the water in the pulp and sand first runs from the lower end of the funnel and after a few seconds agar begins to appear. Suction is stopped, the water and dilute agar discarded, and the filtration now proceeded with. As the filtration continues the amount of suction should be slightly increased. The agar passes through very rapidly, and with the apparatus figured five litres can be filtered in thirty to forty minutes. It is not advisable to filter any greater quantity without changing the paper pulp and sand. For larger quantities of medium, a one- or two-gallon cylindrical stone jar with a false bottom is used, and amounts of agar up to fifty litres an hour can be dealt with.

This agar-broth medium is known as Nutrient Agar or "Ordinary Agar."

When fluid, it is perfectly clear, but when solidified is faintly opalescent. Its advantage is that it is solid at 37° C. which is the optimum temperature for most pathogenic organisms. *The medium must be heated to 98° C. to melt it, but when melted it may be cooled down to 42° C. before solidifying.* This property is utilised in the preparation of serum-agar and blood-agar (*vide* p. 105). The agar may thus be cooled below the coagulating point of the serum proteins—60° C.—and yet remain fluid so that the serum or blood is present unaltered in the medium.

Agar-agar differs from gelatin inasmuch as it is a carbohydrate and is not generally liquefied by bacteria.

Nutrient agar made from infusion broth is distinguished by a **YELLOW** bead in the container, whereas nutrient agar made from digest broth (Hartley's broth) is identified by a **BLACK** bead.

"HORMONE" AGAR

It has been shown that filtration through paper removes from nutrient media certain factors which are favourable to the growth of bacteria.¹ To obviate this, an agar medium ("hormone" agar) may be prepared as follows :

Ingredients.

Ox or horse heart (minced)	.	.	.	500 grams
Peptone	.	.	.	10 grams
Sodium chloride	.	.	.	5 grams
Agar fibre	.	.	.	25 grams
Water	.	.	.	1000 c.c.

Method.

Dissolve the agar by heating in the autoclave; cool to about 50° C. and add the minced heart. Boil the mixture in a pan over a Bunsen flame for fifteen minutes, stirring well while boiling. Add the peptone slowly and the salt, and boil till they are dissolved (about five minutes). Strain through coarse wire gauze. Adjust the reaction of the fluid to pH 7·8 and steam in the Koch steriliser for thirty minutes. Pour into a large funnel closed at the lower end. Then place the funnel in the boiling Koch steriliser, extinguish the flame and allow to stand overnight, so as to cool slowly. Next morning remove the solidified medium from the funnel, cut off and reject the portion containing deposit and use the rest as agar medium.

Both broth and agar media may be enriched or modified by the addition of various substances.

GLUCOSE BROTH.—Broth + 1 or 2 per cent. of glucose. Since glucose acts as a reducing agent, this medium may be used for the growth of anaerobes.

GLYCEROL BROTH.—Broth to which glycerol is added in the proportion of 5–8 per cent. This medium is sometimes used for the growth of the tubercle bacillus.

Similarly, **GLUCOSE AGAR** is made by the addition of 1–2 per cent. of glucose, and **GLYCEROL AGAR** by adding 5–8 per

¹ Cole and Lloyd, *J. Path. and Bact.*, 1916, vol. 21, p. 267.

cent. of glycerol to ordinary agar. The former is used chiefly for deep stab cultures of anaerobes (*vide* p. 136).

STANDARDISATION OF MEDIA

While many bacteria show vigorous growth within a fairly wide range of reaction as regards acidity or alkalinity, there are others which require the reaction of the medium to be adjusted within narrow limits before multiplication takes place. Moreover, all organisms have a particular reaction at which the growth is optimal.

In order, therefore, to secure the best growth, particularly of the highly parasitic organisms, it is necessary that the adjustment of the reaction should be made as accurately as possible.

Two methods are given here—the one in which the acidity or alkalinity is expressed in terms of the absolute acidity which depends on the hydrogen-ion concentration, and the other in relation to the neutral point of an indicator (phenol phthalein). The former method is the more satisfactory.

STANDARDISATION ACCORDING TO HYDROGEN-ION CONCENTRATION.—The true acidity of any fluid depends on the number of dissociated hydrogen-ions present, and the reaction of the medium is dependent on, and measured by, the hydrogen-ion concentration. The greater the concentration, the more acid the medium.

The standard of hydrogen-ion concentration is N/1 acid (*e.g.* N/1 HCl), which, if fully dissociated, contains 1 gram of hydrogen-ions per litre. Then N/10 is only 1/10, or 10^{-1} , of this concentration, N/10,000 is 10^{-4} , and N/1,000,000 is 10^{-6} , of the standard hydrogen-ion concentration.

The hydrogen-ion concentration is expressed in the form of the "hydrogen exponent" designated pH, which is the logarithm of the concentration as compared with the standard, with the negative sign omitted.

N/10,000 HCl has a concentration of 10^{-4} and is represented thus—pH 4.0, while N/1,000,000, or 10^{-6} , has a pH 6.0.

It is therefore apparent that as the hydrogen exponent (pH) increases, the acidity decreases, and that unit difference of pH means ten times difference in the actual acidity.

Thus N/1000 HCl has a pH 3.0 and is ten times as acid as N/10,000 HCl, of which the pH is 4.0.

$$\text{pH} \propto \frac{1}{\text{concentration of H-ions}} \propto \frac{1}{\text{acidity}}$$

The absolute determination of pH is made by means of the hydrogen electrode, and it is found that the concentration of hydrogen-ions in specially purified water is 10^{-7} at 20°C .—*i.e.* pH 7.0. Here there is an equal amount of H-ions and OH (hydroxyl)-ions and the concentration of each is 10^{-7} . If an acid be added—*e.g.* HCl—it dissociates into H- and Cl-ions, and therefore the concentration of H-ions is increased, with a proportional decrease in the OH-ions. If, on the other hand, a base such as NaOH be added, the dissociation causes an increase in the number of OH-ions, with a corresponding decrease of H-ions, hence a lessened hydrogen-ion concentration. If there are more H-ions than OH-ions the solution is acid. Conversely, if there are more OH-ions than H-ions, the solution is alkaline.

In pure neutral water the concentration of H is 10^{-7} , and that of OH 10^{-7} . In an acid solution the concentration of H is greater than 10^{-7} , while that of OH is less than 10^{-7} . Conversely, in an alkaline solution the concentration of H is less than 10^{-7} and that of OH is more than 10^{-7} . The H-ion concentration of blood plasma has a pH 7.5—*i.e.* the concentration of H-ions is less than 10^{-7} while that of the OH-ions is more than 10^{-7} , so that blood plasma is alkaline. This reaction is about the optimum for the growth of most pathogenic micro-organisms.

It is not practicable to use the hydrogen electrode for general laboratory use, and a simple and satisfactory method has been devised whereby media can be adjusted to any desired pH. The method depends on the fact that when a fluid contains buffer salts such as phosphates, the addition of alkali or acid does not cause an abrupt change in the colour of an indicator. For example, phenol sulphone-phthalein (phenol red) is yellow in acid solution and purplish-pink in alkaline solution. If an alkali be added gradually to an acid phosphate solution containing phenol red, the change in colour will commence at pH 6.8, and the colour will become more purplish-pink, until the final change is reached at pH 8.4; thus the "range" is pH 6.8 – pH 8.4, and as it covers the optimum reaction for culture media—namely, pH 7.4 to 7.8—this particular indicator is used.

Other dyes have their own definite range of pH in which colour change occurs, and there is now available a complete series of indicators which exhibit colour changes between pH 1 and pH 10.

The procedure for adjusting culture media to a definite pH is comparatively simple. Solutions of fixed and known hydrogen-ion concentrations are prepared and to each solution a definite amount of indicator (for this purpose phenol red) is added. The resulting tint is the standard to which the medium must be brought by titration with alkali, and so the amount of alkali to be added per litre may easily be calculated.

Apparatus required:

1. A set of tubes of standard bore containing solutions of known pH to which the indicator has been added. These solutions are made up by mixing N/15 Na_2HPO_4 and N/15 KH_2PO_4 solutions in certain proportions. The tubes with the standard solutions, which have a range of pH 6.6–8.0 at intervals of pH 0.2, can be purchased. Details of their preparation may be found in larger works.

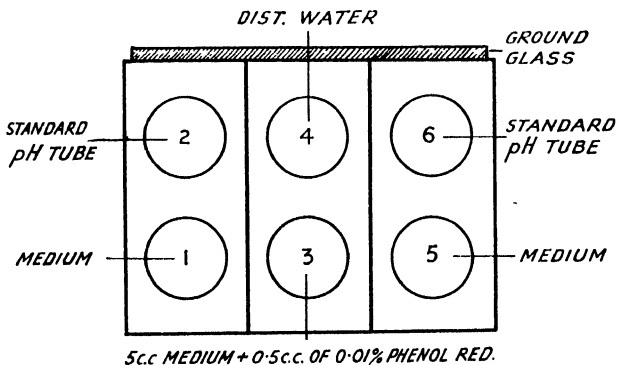
2. A special comparator rack.
3. "Cordite" tubes, which have a uniform thickness of wall and bore, and are identical with the tubes containing the standard solutions.
4. A solution of phenol red, 0.01 per cent., in distilled water.
5. N/20 NaOH made up as follows :—

500 c.c. N/10 NaOH

91 c.c. 0.01 per cent. phenol red
distilled water to 1000 c.c.

6. A burette, preferably a microburette, measuring to 0.01 c.c. (An improvised one may be made from a 1 c.c. graduated pipette, a short piece of rubber tubing, a glass tube drawn out to a fine point, and a pinchcock.)

PLAN OF COMPARATOR RACK



Tube 3 contains 5 c.c. of the medium + 0.5 c.c. of 0.01 per cent. solution of phenol red.

Tubes 1 and 5 contain the medium only.

Tube 4 contains distilled water only.

Tubes 2 and 6 are the standard tubes for comparison. By this arrangement of the tubes the colours of 1 and 2, and of 5 and 6 are superimposed when examined in the rack.

It has been found easier to bring the solution to a tint midway between two standard colours than to make the tint match a given standard. Thus suppose a reaction of pH 7·5 is required, then standard tubes pH 7·4 and pH 7·6 are placed in positions 2 and 6 of the rack.

The N/20 NaOH solution is run into tube 3 until the tint produced is midway between the tints of tubes 2 and 6, and the amount noted. The average of two readings is taken and the calculation is as follows:—

Let the number of c.c. of N/20 NaOH = y .

5 c.c. medium require y c.c. N/20 NaOH
to adjust reaction to pH 7·5

1000 c.c. medium require $200y$ c.c. N/20 NaOH.

1000 c.c. medium require $10y$ c.c. N/1 NaOH.

Example: Suppose $y = 1·15$, then 11·5 c.c. N/1 NaOH are required per litre of the medium to adjust the reaction to pH 7·5.

It will readily be seen that the tint due to the mixture of the medium and the indicator in tube 3 is compensated for by the medium in tubes 1 and 5.

The indicator is incorporated in the standard alkali solution, so that when the medium in tube 3 is titrated, the actual concentration of the dye always remains constant.

It is preferable when making media in bulk to have the reaction distinctly alkaline, and to adjust it for use by the addition of acid. The addition of alkali to an acid medium causes a precipitation of phosphates and the medium has to be filtered again before use. If, however, the medium be made distinctly alkaline, acid has to be added to obtain the correct reaction; no precipitate occurs and the medium is perfectly clear. In this case the titration with the standard pH tubes is carried out in exactly the same manner, except that, instead of caustic soda solution, N/20 HCl containing the indicator is employed, and the calculated amount of normal hydrochloric acid is added per litre to obtain the desired reaction.

The standardisation of a solid medium such as nutrient agar presents greater difficulty than in the

case of fluid media. The medium may be titrated when liquid, but the exact determination is not easy to obtain with any degree of accuracy. It has been found that agar of good quality has very little effect on the reaction of the broth to which it is added, but the reaction of the finished agar should be controlled by titrating the melted medium and then comparing the colour when cold. Gelatin may conveniently be adjusted if the medium is liquefied and kept at about 37°C .

Other indicators used for bacteriological work are brom-cresol-purple, which changes colour from yellow to purple over the range pH 5.2-6.8, and brom-thymol-blue, which has a colour range from yellow to blue between pH 6.0-7.6.

EYRE'S METHOD.—In this method the reaction is expressed as being acid or alkaline in terms of the neutral point of phenol phthalein. If acid, the prefix + is employed, while the sign — denotes alkalinity. The precise reaction is expressed by the number of c.c. of normal acid or normal alkali that are required per litre of medium in order to adjust it to the neutral point of phenol phthalein. Thus a reaction of + 20 indicates that the medium is acid (by the +) and that it requires 20 c.c. of normal caustic soda (N/1 NaOH) per litre in order to make it neutral to phenol phthalein. Similarly a reaction of -8 shows that the medium is alkaline and requires 8 c.c. of normal acid (N/1 HCl) per litre in order to render it neutral.

For the growth of most parasitic bacteria, the optimum reaction is about + 10—*i.e.* acid to phenol phthalein.

Media were originally standardised by rendering them slightly alkaline to litmus. Litmus and phenol phthalein have not the same neutral point, and the neutral point of litmus is acid to phenol phthalein. The optimum reaction of media in general lies between the neutral points of these two indicators—*i.e.* acid to phenol phthalein and alkaline to litmus.

Method.—Place 20 c.c. of broth in an evaporating dish and add 20 c.c. of distilled water and a few drops of 0.5 per cent. solution of phenol phthalein in alcohol. As the mixture must be heated to remove carbon dioxide, the evaporating dish should be placed on a water-bath, or warmed (on a tripod stand) over a Bunsen burner.

Decinormal caustic soda (N/10 NaOH) is run in from a burette until the neutral point, as indicated by a faint but permanent trace of pink, is reached. Note the amount of

alkali added, repeat the titration, and take the mean of the two readings. The calculation is as follows :—

Suppose y is this mean reading (in c.c. N/10 NaOH).
Then

20 c.c. of medium require y c.c. N/10 NaOH
to make it neutral to phenol phthalein.
1000 c.c. of medium require $50y$ c.c. N/10 NaOH
or $5y$ c.c. N/1 NaOH
to make it neutral to phenol phthalein.

The reaction is therefore $+ 5y$.

Example : Suppose $y = 5.2$ c.c., then the reaction is $+ 26$.

26 c.c. of N/1 NaOH would therefore make 1000 c.c. *neutral* to phenol phthalein, but the required reaction is $+ 10$, so that $26 - 10$ c.c.—i.e. 16 c.c. of N/1 NaOH—must be added per litre to give this reaction.

While $+ 10$ is a suitable reaction for most organisms, some bacteria, such as the gonococcus and meningococcus, grow best in a slightly more alkaline medium having a reaction of $+ 6$.

The disadvantage of Eyre's method is that, while it seems sound in theory, the practical application shows a wide margin of error. This is due to the fact that the "end-point" of the indicator is not sharp owing to the presence of phosphates in the medium. These act as "buffer salts," so that near the neutral point the addition of acid or alkali produces only a gradual change in the colour of the medium. The precise end-point cannot be determined with any degree of accuracy, as the personal factor predominates, depending on the colour sense of the manipulator and what he considers an adequate colour change.

PEPTONE WATER

This is a simple medium, consisting of

Peptone 1 per cent.

NaCl 0.5 „

dissolved in warm water and then filtered. It is sterilised in the autoclave. It is used chiefly as the basis for sugar-fermentation media, since broth and nutrient agar, being made from meat, contain a small amount of muscle sugar, and it is essential that the basic medium, to which various carbohydrates are added for fermentation tests, should be free from natural sugar.

Peptone water is best made up in bulk and distributed in 250 c.c. amounts in 12 oz. screw-capped bottles. The caps are tightly screwed on and the whole batch of bottles is sterilised in the autoclave at 5 lbs. pressure for thirty minutes. The peptone water is later distributed into tubes or small bottles as required. Plain peptone water *without indicator* is distinguished by a WHITE bead in the container, while peptone water *with indicator* is identified by a BROWN bead.

Peptone water is used to test the formation of indole (*vide* p. 324), and also for the enrichment of the *Vibrio cholerae*, when isolating this organism from infected material. In the latter case the medium should be adjusted to a reaction neutral to phenol phthalein (approximately pH 8.4), as *V. cholerae* prefers an alkaline medium.

PEPTONE WATER AGAR.—This consists of peptone water solidified with 2 per cent. agar, and is used as a basis for solid media containing sugars (*vide* p. 252).

SUGAR MEDIA

Under the designation of “sugars” are included a variety of fermentable substances, chiefly carbohydrates, which are used in the identification and classification of organisms (*vide* p. 100).

These substances are fermented with the formation of acid, and in many cases gas is formed in addition.

The medium consists of peptone water, to which the fermentable substance is added in the proportion of 0.5 or 1 per cent. An indicator is added to denote acid change. This may be Kubel-Tiemann litmus solution (now rarely used), neutral red (0.25 per cent. of a 1 per cent. solution), or Andrade's indicator (1 per cent.).¹ If acid is produced, the litmus turns bright red; the neutral red, pink; and Andrade's indicator, reddish pink. In order to detect gas, a small inverted tube is placed in each culture tube (Durham's fermentation tube). During the process of sterilisation the heat drives out the air from the inverted tubes, which

¹ Made by adding sodium hydrate to a 0.5 per cent. solution of acid fuchsin until the colour just becomes yellow.

when cool should be completely filled with liquid and contain no air bubbles.

The original method of making the medium is as follows :—

The stoppered test-tubes containing the small inverted tubes are sterilised by dry heat in the hot-air oven. The peptone water (with the indicator added) is sterilised by autoclaving. The sugars are made up separately in 10 per cent. solutions in distilled water, which are sterilised in the steamer or by filtration. The requisite amount of sugar solution is added to the peptone water. The medium is tubed (*vide* p. 124), and steamed for twenty minutes on three successive days.

The various sugar media in tubes can be distinguished by having the cotton-wool stoppers of different colours. It is better to employ wool dyed in bulk rather than to colour white-wool stoppers with various stains.

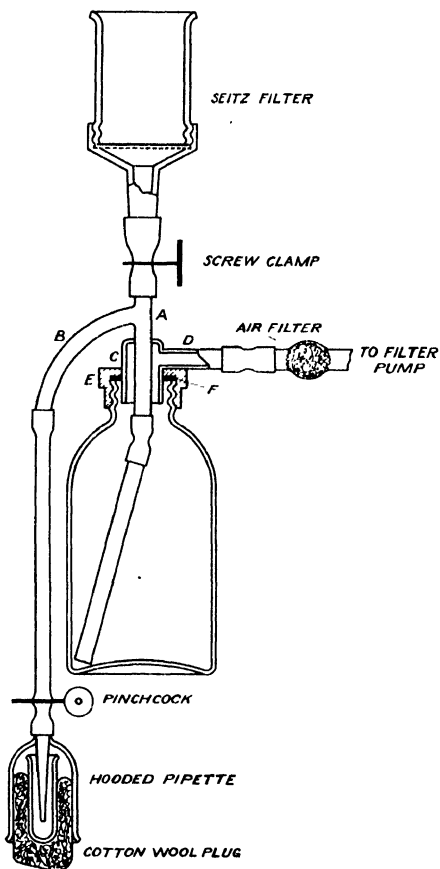
The following alternative method of making sugar media is recommended :

Peptone water with an indicator is tubed, the Durham fermentation tube inserted, and the test-tubes stoppered with coloured cotton-wool. They are then sterilised in the steam steriliser, or in the autoclave for half-an-hour at 5 lbs. pressure. The sugars are made up separately in 10 per cent. solutions in distilled water, and are sterilised, preferably by filtration (Seitz filter), or in the steamer. The sterile sugars are kept conveniently in 10 oz. screw-neck bottles fitted with a siphon and hooded pipette, as shown in the diagram (p. 98).

This method of obtaining small quantities of sterile fluid from bulk can be applied to serum as well as to sugars (*vide* p. 103).

The apparatus recommended consists of a special metal fitting adapted to a 10 or 12 oz. white "medical flat" bottle with a screw neck. It consists of a straight piece of tube (A), with a curved side arm (B); around this is a slightly larger tube (C), with a side arm (D), and fitted with a screw cap (E), which screws on to the bottle, a rubber washer (F) ensuring an air-tight joint. To the upper end of tube (A) is connected a Seitz filter by means of a short piece of pressure tubing furnished with a screw clamp, while attached to the lower end,

by means of a short piece of rubber tubing, is a glass tube, 5 mm. in diameter, reaching to the bottom of the bottle. To the side arm (B) is connected a piece of rubber tubing furnished



at the other end with a pinchcock and hooded pipette. The hooded pipette is closed by a coloured cotton-wool stopper¹ containing a small glass test-tube to cover the delivery tube. The side arm (D) is connected by pressure tubing to a cotton

¹ The colour denotes the particular sugar used.

wool air filter, the other end of which is attached to a filter pump.

The whole apparatus, as figured, is connected up (the joints being bound by copper wire), wrapped in kraft paper, and sterilised in the autoclave.

Filtration of the sugar is accomplished in the usual manner, and before the pressure is released, the pressure tubing to the Seitz filter is closed by means of the screw clamp. The filter is then removed, and the end of the pressure tubing plugged with coloured wool. The filter pump is now disconnected, when the air pressure forces the solution down the siphon tube as far as the pinchcock, so that the siphon is in operation as soon as the pinchcock is opened. Alternatively air may be forced through the cotton-wool filter on D by means of a rubber blowball to start the siphon action. In use the neck of the bottle is held by a clamp at the top of a tall retort stand. The stem of the hooded pipette is held below the bottle by means of another clamp, at a height convenient for placing a test-tube under it to receive the sugar solution. The coloured-wool stopper is removed and the inside of the pipette flamed. After use the stopper is replaced and the hooded pipette fastened to the neck of the bottle by a piece of copper wire. The number of drops per c.c. delivered from the pipette is determined, so that the amount of sugar solution required for the volume of peptone water is easily estimated. Thus, if a pipette delivers 18 drops per c.c., then 9 drops (0.5 c.c.) of 10 per cent. sugar solution per tube of 5 c.c. peptone water give a final concentration of 1 per cent. sugar.

It is recommended that sugar media as above be distributed in 5 c.c. amounts into small screw-capped bottles, p. 126, instead of test-tubes. By this means the medium can be stored without risk of contamination or alteration in the concentration of the ingredients. This is particularly useful in the case of certain sugars which are only occasionally required. Moreover, sugar media in these bottles can easily be transported without leakage or spilling. As a result of shaking during transit, air may enter the Durham fermentation tube, but it is easily removed. The bottle is merely inverted, the Durham tube drops into the neck of the bottle, and the amount of fluid is such that the open end of the tube is below the surface. The bubble of air escapes and on turning the bottle to the proper position the Durham tube falls to the bottom of the bottle full of liquid and without any air.

In order to identify the various sugar media the caps are painted with "Luc" cellulose paint, of which many colours are available. The screw caps, as received from the makers, are already sprayed white, and the added colours on the tops of the small screw-capped bottles indicate the same sugars as

the coloured cotton-wool plugs. Thus, in the London County Council laboratories green = glucose, red = lactose, mauve = mannite, etc.

After the bottles have been prepared, the appropriate colour is painted on the caps. A batch of a gross takes only a few minutes, and the paint is dry within thirty minutes. When two colours are used together for identifying media—e.g. yellow and white for inulin—only one half of the white cap is painted.

The fermentable substances most commonly used are the following :—

Monosaccharides :

(a) *Pentoses* —

- (1) Arabinose (from gum arabic).
- (2) Xylose (from wood gum).
- (3) Rhamnose.

(b) *Hexoses*—

- (1) Glucose (Dextrose, or Grape sugar).
- (2) Lactulose.
- (3) Mannose (from the ivory nut).
- (4) Galactose (made by the hydrolysis of lactose).

Disaccharides :

- (1) Saccharose (Cane Sugar).
- (2) Maltose (Malt Sugar).
- (3) Lactose (Milk Sugar).

Trisaccharide :

Raffinose.

Polysaccharides :

- (1) Starch.
- (2) Inulin (from dahlia tubers).
- (3) Dextrin.
- (4) Glycogen.

Alcohols :

- (a) *Trihydric*.—Glycerol (Glycerin).
- (b) *Tetrahydric*.—Erythritol (Erythrite).
- (c) *Pentahydric*.—Adonitol (Adonite).
- (d) *Hexahydric*.—(1) Mannitol (Mannite).
(2) Dulcitol (Dulcite).
(3) Sorbitol (Sorbite).

Glucosides (vegetable products which on hydrolysis yield a sugar) :

- (1) Salicin.
- (2) Coniferin.
- (3) Aesculin.

Non-carbohydrate Substances :

Inosite—a benzol compound.

LITMUS MILK

Used in testing for the fermentation of lactose and clotting of milk.

Fresh milk is steamed for twenty minutes and then allowed to stand for twenty-four hours in order that the cream may separate. The milk is siphoned off and coloured with litmus. Amounts of 6 c.c. are placed in tubes, which are then sterilised by steaming for twenty minutes each day on three successive days. As this medium is not often used, it is recommended that it be distributed in 5 c.c. amounts in small screw-capped bottles. These are sterilised as above, with the cap tightly screwed on.

SERUM AND BLOOD MEDIA

These may be divided into two classes :

- (1) Where the serum or blood is coagulated by heat (above 60°C.) and a solid medium results.
- (2) Where the serum or blood is added in fluid form to enrich simpler media.

MEDIA CONSISTING ALMOST ENTIRELY OF SERUM OR BLOOD

LÖFFLER'S BLOOD SERUM.—To ox, sheep or horse serum is added one-third of its volume of 1 per cent. glucose-broth. The mixture is added to stoppered sterilised tubes, which are laid on a sloped tray and placed in the serum inspissator. The temperature is then slowly raised to 75°C. and maintained for six hours, when the serum coagulates to a yellowish white solid. The tubes are thereafter sterilised at 90°C. (in the top of the steam steriliser) for twenty minutes on each of three successive days. If sterile serum is used, only four hours' inspissation at 75°C. is necessary. It is essential that over-heating be avoided, as the expansion of air bubbles and the formation of steam from the fluid droplets in the partially solidified material lead to the disruption of the medium.

We specially advocate that Löffler's medium be made in the small screw-capped bottles. The requisite number of bottles fitted with caps (as received from the makers) are autoclaved

at 15 lbs. pressure for twenty minutes. The serum-glucose-broth mixture is added to the bottles in 5 c.c. amounts under sterile conditions. The caps are then tightly screwed on, and the bottles carefully laid in a horizontal position in the inspissator. The temperature is slowly raised to 75° C. and maintained for four hours. The culture medium should be allowed to cool before being handled. If the serum-glucose-broth mixture is not completely sterile, the bottles after coagulation of the medium are placed in the steamer the next day for one hour.

In these containers Löffler's serum is most useful for diphtheria diagnosis in schools, clinics, small hospitals, etc., where cultures are made only from time to time. The medium can be stored for long periods, and the small amount of water of condensation present keeps the surface constantly moist. A very profuse growth occurs after incubation for a few hours. The use of the small bottles is thus most economical.

Löffler's serum is especially useful for the growth of the diphtheria bacillus. Not only does it produce a luxuriant growth in a short time (twelve to eighteen hours), but it is also valuable in eliciting the characteristic staining reaction of the organism by Neisser's method (*vide* p. 160).

Collection of Blood. -- A sterile wide-mouthed stoppered bottle is taken to the abattoir at a time when animals, preferably sheep, are being killed. After the neck vessels have been severed, the blood is allowed to flow for some time and then the stream from the carotid artery is allowed to spurt directly into the bottle. When filled, the bottle is stoppered and returned carefully to the laboratory. The clot is then separated from the sides of the bottle by means of a stiff sterile wire. The blood is kept overnight in the ice-chest and the clear serum pipetted off. With care, contamination can be avoided.

Defibrinated blood is collected in a similar way. The stoppered bottle, however, contains glass beads. The bottle is only half filled, and immediately a sufficient quantity of blood has been collected the stopper is replaced and the bottle continuously shaken for about five minutes. The blood so treated does not clot on standing.

Another useful method is the following :

Horse blood is obtained from the slaughter-house, the horse being bled directly into jars containing 10 c.c. of a 10 per cent. solution of neutral potassium oxalate per litre of blood. The red corpuscles are allowed to settle overnight in the cold and the plasma is siphoned off into a Winchester quart bottle ; 22.5 c.c. of a 4 per cent. solution of calcium chloride per litre of plasma are added, and the bottle is shaken immediately on a machine until the fibrin has separated. The serum is now filtered through a Seitz bacterial filter (14 cm. diameter film) into a large sterile jar or filter flask of 1.5 litres capacity, fitted with siphon delivery tube and hooded pipette, as described on p. 97. The serum is stored in the cold-chamber and used as required.

Sterile specimens can be obtained by inserting a cannula or wide-bore needle into the external jugular vein. If a sheep is selected, the wool is clipped from the side of the neck and the part shaved. Contamination can be minimised by placing a bag made of waterproof material over the head of the animal. It is best to use a cannula connected by rubber tubing to a screw-capped bottle (*vide* p. 79), the whole being enclosed in kraft paper and sterilised. The vein may be made prominent by pressure on the lower part of the side of the neck. The skin over the vein is carefully sterilised with soap and water and then alcohol. The cannula is inserted into the vein and the requisite amount of blood removed. Horses are treated similarly except that it is advisable to make a small incision with a sharp knife in the skin over the vein. The cannula is then more easily introduced.

The sterile defibrinated horse blood is immediately distributed in 10 c.c. amounts into sterile small 1 oz. screw-capped bottles (p. 126) and stored in the cold-room. In smaller laboratories amounts of 5 c.c. will probably suffice. The defibrinated blood thus stored will keep for periods up to three months.

INSPISSATOR

This apparatus is used for the manufacture of Löffler's serum medium, and Dorset's egg medium (*vide infra*). It consists of a water-jacketed copper box, the temperature of which can be regulated automatically. The serum or egg medium is tubed and placed in special racks, so that the tubes are at the correct angle for forming slopes. The temperature used for serum is 75° C., while that for egg medium is 80° C. At these temperatures the protein material is completely solidified, but the temperature is not so high as to cause bubbles of steam to disrupt the surface of the medium. As the medium is apt to dry if kept in

the inspissator for any time, a small opening should be present in the inner wall communicating with the top of the water-chamber above the level of the water. Water-vapour can enter the interior of the inspissator and the medium is kept moist. Electric inspissators without a water-jacket do not make such satisfactory media.

Hiss's SERUM WATER.—As certain pathogenic organisms—*e.g.* streptococcus, pneumococcus—will not grow well in ordinary sugar media, it is necessary for fermentation tests to use a medium containing serum.

One part of serum is mixed with three parts of distilled water, and 1 per cent. of Andrade's indicator is added. (Some workers prefer to substitute 1 per cent. peptone water for the distilled water.) The various sugars are incorporated in the proportion of 1 per cent. This medium, if not acid, does not coagulate on heating, and may be sterilised in the steamer in the same way as other sugar media—namely, twenty minutes each day on three successive days.

Alternatively, the mixture of serum, distilled water and indicator is sterilised in the steamer, and the appropriate amount of the requisite sugar is added as described on p. 99.

Some workers prefer to use phenol red as the indicator, and to adjust the reaction of the medium to a suitable pH—*e.g.* 7.6—before sterilisation.

Fermentation is indicated by the production of acid, which alters the indicator and causes coagulation of the medium.

Small screw-capped bottles may be used most conveniently as containers for this medium. The method recommended is similar to that used for peptone-water sugar media, p. 96.

The Hiss's serum water is made up with indicator, but without any carbohydrate added. The medium is distributed in 5 c.c. amounts (or less if desired) in 1 oz. bottles. The caps are tightly screwed on, and the whole batch is sterilised in the steamer for twenty minutes on three consecutive days. When a batch of any particular carbohydrate medium is required, the requisite amount of sterile 10 per cent. sugar is added from the siphon-filter bottle described on p. 98. The caps are then painted with "Luc" cellulose paint according to the

sugar used (*vide* p. 99). Alternatively, small quantities of the rarer sugars in 10 per cent. aqueous solutions may be stored in screw-capped bottles fitted with a perforated cap and rubber washer similar to the blood-culture bottle described on p. 142. When the sugar solution is required it is withdrawn from the bottle by perforating the rubber with the needle of a sterile syringe in exactly the same manner as the rubber cap of a vaccine bottle. Contamination will not occur if the procedure is carried out carefully and under a hood, and the medium with the sugar added keeps as long as that sterilised in a tightly closed container.

MEDIA ENRICHED WITH SERUM OR BLOOD

These media are used for certain delicate pathogens, such as the pneumococcus, gonococcus and meningococcus, which grow feebly or not at all on ordinary media.

SERUM-AGAR.—Ordinary nutrient agar containing 2 per cent. agar + 5 per cent. of sterile uncoagulated serum. Animal serum is quite satisfactory, and can be obtained in the laboratory by bleeding a rabbit (*vide infra*), allowing the blood to coagulate in a sterile stoppered measuring cylinder, and removing the serum after it has fully separated. All the necessary precautions must be taken to avoid contamination. Serum can be stored in sealed tubes after heating at 57° C. on three successive days, but fresh serum yields much better results than heated serum in the culture of certain pathogens—*e.g.* gonococcus.

Horse serum kept in a sterile bottle, as described on p. 103, is recommended where large quantities of serum-agar are used.

The agar is first melted and then cooled to about 50° C. The serum is usually added to the agar in tubes, and, after it is incorporated, the medium is either solidified in the form of slopes, or poured into Petri dishes (*vide* p. 127). As serum-agar is indistinguishable from ordinary agar, the tube (or dish) should be marked “+ S,” or plugged with coloured wool.

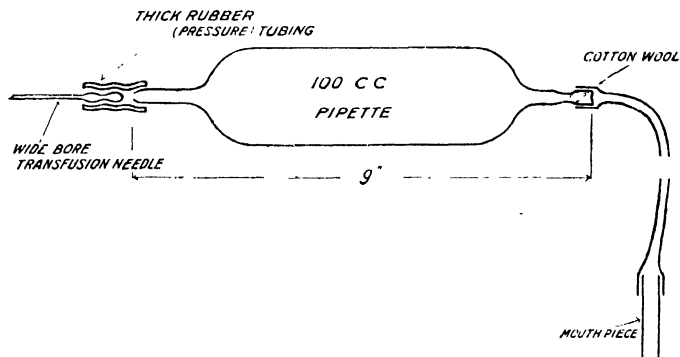
Sterile hydrocele fluid or sterile ascitic fluid, withdrawn aseptically, may be used instead of serum.

SERUM-SMEARED AGAR.—This is made by running a few drops of sterile serum on the surface of an agar slope or plate (*vide* p. 127). This medium is not so satisfactory as the preceding one, but is useful in an emergency.

BLOOD-AGAR.—This is an important medium and is specially suitable for the gonococcus, the haemophilic group of bacteria (e.g. *B. influenzae*) and other delicate pathogens.

Either human or animal blood is suitable. Defibrinated rabbit blood obtained from the ear vein or by cardiac puncture can be recommended for general use.

20 to 30 c.c. of blood may be obtained easily from the ear vein of a large rabbit without distress to the animal. The ear is



shaved and sterilised with sterile gauze soaked in spirit. Meanwhile a small vessel containing vaseline has been heated over the Bunsen to render it sterile, and when cool, but still fluid, the vaseline is painted over the vein, and on the margin and under side of the ear. The ear is held forward and the vein is made prominent by means of a small spring clip at the base of the ear, and then incised with a small sharp sterile scalpel. The blood flows over the vaseline, and is allowed to drop into a sterile flask containing glass beads (*vide infra*). The vessels of the ear can be dilated by rubbing the part not covered by vaseline with a

pledget of wool moistened with xylol. When sufficient blood has been obtained the clip is removed and a piece of cotton-wool pressed firmly over the cut in the vein. The xylol is removed from the ear with spirit, and some vaseline then lightly smeared on. Water should always be provided in the cage of the animal after bleeding. This is a very useful method for obtaining small quantities of sterile rabbit blood.

For cardiac puncture the procedure is as follows :—

The animal is fastened to a board and the fur clipped over the left side of the chest ; the area is shaved and then sterilised with alcohol and ether. A 100 c.c. bulb pipette (see diagram) is cut down at both ends to 9 in. in length, one end being slightly tapered and the other end stoppered with cotton-wool. It is wrapped in kraft paper and sterilised in the hot-air chamber. A wide-bore transfusion needle is fitted into a short length ($1\frac{1}{2}$ in.) of thick rubber tubing and sterilised by boiling. When the animal is anaesthetised, the rubber tubing is attached to the tapered end of the pipette and to the other end is fitted a mouth-piece such as that used in pipetting (*vide* p. 198). The needle is inserted into the left side of the chest and suction applied. The needle should lie in the right ventricle of the heart, and blood rapidly flows into the pipette. About 50 c.c. of blood per kilo. of body-weight can be obtained. The blood is then transferred to a sterile 500 c.c. flask containing glass beads. Agitation should be kept up for at least five minutes, to ensure that all the fibrin is separated.

Human blood may be obtained easily by means of vein puncture (see blood culture, p.141).

The blood is added to melted 2 per cent. agar at 50° C. in the proportion of 5-10 per cent., as in preparing serum-agar (*q.v.*).

A considerable saving of blood can be effected if blood-agar plates are made in the following manner :—

Pour a thin layer (about 5 c.c.) of melted plain agar into a Petri dish and allow to set. Make 10 per cent. blood-agar by adding defibrinated blood to melted agar at 55° C., and pour a similar quantity on the surface of the agar in the dish and allow it to set. Since the surface layer only is utilised for growth, blood is not required in the lower half of the dish. A fairly thick layer of medium is required to prevent excessive drying during incubation. If this were entirely 10 per cent. blood-agar, the medium would be almost opaque when viewed by transmitted light through the dish, and methaemoglobin formation by organisms would be difficult to see. Moreover, haemolysis would not be easy to determine in young cultures

if a thick blood-agar layer were present. The method of the double layer of agar and blood-agar is not only more economical, but it forms a bright, light-transmitting medium on which methaemoglobin formation and haemolysis can easily be observed. If only one plate is required, two 5 c.c. amounts of agar are melted. The contents of one is poured into the Petri dish as the first layer. The contents of the other are cooled to 55° C. and 0.5 c.c. blood added. After mixing, this is poured on the surface of the first layer in the dish and allowed to cool.

It is advisable however to prepare several plates at one time as follows :—

Two 100 c.c. screw-capped bottles of agar (p. 79), 10 c.c. defibrinated horse blood in a 1 oz. screw-capped bottle (p. 126), and twelve sterile Petri dishes are required. The agar is melted in the steriliser and the contents of one bottle are distributed into the Petri dishes. While this agar is setting, the other bottle is cooled to 55° C. The cap is unscrewed and the 10 c.c. of blood poured in from the 1 oz. screw-capped bottle. No pipette is necessary as the screw cap keeps the lip of the bottle sterile. The cap is again screwed on, the bottle inverted several times to mix thoroughly the agar and blood. The blood-agar is now distributed into the Petri dishes on the surface of the first layer of agar. Any bubbles caused by the mixing can easily be removed by drawing a Bunsen flame quickly across the surface of the medium in the dish. Two 50 c.c. amounts of agar and 5 c.c. of blood will make six plates.

For special purposes, amounts of blood up to 50 per cent. may be added.

Note.—For laboratories where it is inconvenient to obtain rabbit or human blood, it should be noted that Messrs Burroughs Wellcome Ltd. supply sterile defibrinated horse blood.

HEATED-BLOOD-AGAR ("Chocolate Agar")

This medium is suitable for cultivating *B. influenzae* and certain other organisms, such as the pneumococcus. To 5 c.c. melted heart-digest agar medium at 60° C. add 0.5 c.c. (9 or 10 drops) of defibrinated rabbit blood. Heat the mixture by immersing the tube for exactly one minute in boiling water, and allow the medium to solidify in the sloped position. If a plate is required, 10 c.c. of agar and 1 c.c. of blood are used.

DORSET'S EGG MEDIUM

This medium is used for growing the tubercle bacillus. Four "new laid" eggs are beaten up and 25 c.c. distilled water then added. The mixture is strained through muslin to remove air bubbles, run into sterile tubes (*vide* p. 124), and solidified in the sloped position in the serum inspissator at 75°–80° C. The tubes are then sterilised at 90° C. (at the top of the steam steriliser) for twenty minutes each day on three successive days.

All apparatus used should be sterile, and the eggs, before they are broken, should be placed for a few minutes in alcohol; on removal the alcohol is allowed to evaporate.

The addition of sufficient basic fuchsin to the medium to render it pale pink is advisable, as early growths of the tubercle bacillus are thus more easily seen.

As the tubercle bacillus may take some weeks to grow, the tubes are sealed after inoculation by pushing down the cotton-wool stopper below the top of the tube, and pouring in a little melted paraffin wax, or they can be covered with a rubber cap. We advocate however that all media for growth of the tubercle bacillus be distributed into small screw-capped bottles as described on p. 126.

A useful *modification of Dorset's medium* is the following:—

Break into a sterile bowl fresh eggs which have been washed in soap and water and then dried, and beat thoroughly with a sterile knife to mix the yolks and whites. Strain the mixture through sterile cheese-cloth over a filter funnel, and to every 75 c.c. of egg mixture (usually two eggs) add 25 c.c. of sterile digest broth and 1 c.c. of a 1 per cent. aqueous solution of crystal violet. Tube the medium into sterile test-tubes (avoiding the formation of air bubbles) and coagulate in the sloped position in the inspissator at 80° C. Sterilise by heating in the inspissator at 80° C. for two hours on three successive days. If drying is prevented the medium keeps for several months, but if the surface is dry a few drops of sterile water are placed on the medium before inoculation. The material to be inoculated should be well rubbed over the surface of the medium.

GLYCEROL EGG MEDIUM (for growing the human type of tubercle bacillus) is prepared as above, but with the addition of 6 per cent. of glycerol to the digest broth and egg mixture.

LÖWENSTEIN-JENSEN MEDIUM FOR THE CULTIVATION AND DIFFERENTIATION OF HUMAN AND BOVINE TYPES OF THE TUBERCLE BACILLUS

(1) *Mineral Salt Solution.*¹

Potassium dihydrogen phosphate	
KH_2PO_4 (A.R.)	0.4 per cent.
Magnesium sulphate (A.R.)	0.04 „ „
Magnesium citrate	0.1 „ „
Asparagin	0.6 „ „
Glycerol (A.R.)	2.0 „ „
Distilled water.	

Heat to dissolve.

The solution is boiled or placed in the steamer for two hours and allowed to cool overnight. 600 c.c. is a convenient quantity to prepare.

(2) *Salt-Starch Solution.*

To each 600 c.c. are now added 30 grams of potato starch.¹ This is mixed and heated in a water-bath with constant stirring for fifteen to twenty minutes, until a satisfactory paste is produced, and then allowed to remain for one hour in a water-bath at 56° C.

(3) *Egg Fluid.*

Hens' eggs are washed, to disinfect them thoroughly, in a 5 per cent. soft-soap solution, after which they are left in running water for one to two hours. The eggs must be less than one week old, and 20 eggs are usually sufficient for 1 litre of egg fluid, but if they are small, 22 eggs should be used. The eggs are now broken one by one into a sterile cup, the yolk and white mixed, and poured into a sterile vessel.

For each 600 c.c. of salt-starch solution, 1 litre of egg fluid is required. The egg fluid is mixed very thoroughly for ten minutes (or shaken in a machine), after which it is filtered through sterile gauze, and to it is added the salt-starch solution at the termination of one hour in the water-bath at 56° C.

(4) *Malachite Green Solution.*

Make a 2 per cent. solution of malachite green in distilled water and incubate for one to two hours. To each 1600 c.c. of prepared substance (1 litre egg fluid + 600 c.c. salt-starch solution) add 20 c.c. of 2 per cent. malachite green.

The medium is now tubed in 5 c.c. amounts in 1 oz.

¹ The chemicals used by us are obtained from British Drug Houses, Ltd., and where specified, the A.R. (Analytical Reagent) product should be used.

bottles (p. 126) and the caps tightly screwed on. The bottles are laid horizontally in the inspissator and are heated at 85° C. for half-an-hour. They are allowed to remain in the inspissator overnight, and are heated again the following day at 75° C. for half-an-hour.

In the screw-capped bottles the medium will keep for many months, but if slopes are made in test-tubes they must be stored in the cold and used within a month.

On this medium the human type of tubercle bacillus grows very luxuriantly, in the form of large heaped-up dry yellow colonies, while the bovine type shows small discrete colourless colonies.

This medium will show good primary growth of tubercle bacilli (*e.g.* from sputum after treatment with 1 per cent. caustic soda, *vide* p. 302) in ten to twelve days. It can be strongly recommended, particularly for the human type.

POTATO SLOPES

This medium is not often used. Large potatoes are selected, carefully washed and peeled. By means of a potato borer (or large cork borer) a cylinder of potato is obtained, which is then washed in running water to remove excess of starch. This is cut obliquely and each half is placed in a test-tube with the thick end resting on a plug of cotton-wool, or in a special potato tube. The tubes are then filled with sterile water and placed in the steam steriliser for half-an-hour. The water is poured off and the tubes autoclaved at 10 lbs. pressure for twenty minutes.

ALKALINE POTATO MEDIUM.—Prepared as above, but instead of filling the tubes with water, 0·7 per cent. sodium bicarbonate solution is added. The subsequent treatment is the same.

GLYCEROL POTATO.—Also prepared as above, 6 per cent. solution of glycerol being used instead of water. This medium has been used for the cultivation of the tubercle bacillus.

✓ MEDIA FOR SPECIAL PURPOSES

MACCONKEY'S BILE-SALT NEUTRAL-RED LACTOSE AGAR

This is a valuable medium for differentiating intestinal organisms of the coli-typhoid group. It is a peptone solution solidified with agar, to which bile-salt, 0·5 per cent., and lactose, 1 per cent., are added, with neutral red as the indicator.

<i>Add</i>	Peptone	2 per cent.
	Sodium taurocholate (commercial)	0.5 „

to tap water and dissolve by heat. Then add 2 per cent. agar and dissolve in the steamer or autoclave. Filter, and clear with white of egg. Add a sufficient amount (about 1:400) of a freshly prepared 1 per cent. solution of neutral red to give the medium a distinct reddish-brown colour. If the medium is acid, and assumes a rose-pink colour, add caustic soda solution until the colour becomes definitely reddish-brown. The medium is then sterilised in the steamer and finally 1 per cent. lactose (previously sterilised separately in a 10 per cent. watery solution) is incorporated. The completed medium may be sterilised as in the case of other sugar media.

Organisms which produce acid from lactose—e.g. *B. coli*—form rose-pink-coloured colonies, whereas the colonies of non-lactose-fermenters—e.g. *B. typhosus*—are colourless.

MacConkey's medium immediately after being filtered should be bottled in 100 c.c. and 250 c.c. amounts in 6 oz. and 12 oz. screw-capped "medical flats." It is then sterilised as above. The 100 c.c. of media when melted will be sufficient for seven Petri plates, while 250 c.c. will make eighteen.

When MacConkey's medium is stored for any length of time the neutral-red indicator tends to fade. In order to overcome this, the medium is made up in the usual way but without neutral red, and the pH is adjusted so that the correct shade of colour is obtained when the indicator is added. The MacConkey agar without indicator is indistinguishable from ordinary nutrient agar, but is identified by a RED bead (p. 80). The neutral red is made up in a 2 per cent. alcoholic solution and 0.3 c.c. per 100 c.c. of medium is the average quantity used. When plates are to be poured the bottle of MacConkey agar is melted, the indicator added, the screw-cap replaced, and the contents thoroughly mixed before pouring.

ENDO'S MEDIUM (ROBINSON AND RETTGER'S MODIFICATION)

Distilled water, 1 litre; agar, 25 grams; beef extract (Lab. Lemco), 5 grams.; peptone, 10 grams. Add the water

to the agar, reserving 10 per cent. to dissolve the other ingredients. Dissolve the agar by heating in the autoclave at 15 lbs. for thirty minutes. Dissolve the peptone and meat extract by heating in the water-bath. Combine the two parts and make up to 1 litre. Adjust to pH 8·5 with 10 per cent. sodium carbonate solution, or adjust first to pH 7 and then add 10 c.c. of 10 per cent. sodium carbonate. Filter when hot. Bottle and sterilise in the autoclave.

When required for use melt the agar, and to each 100 c.c. add 10 c.c. 10 per cent. solution of lactose, 0·26 c.c. saturated alcoholic solution of basic fuchsin, and 1 c.c. 10 per cent. solution of sodium bisulphite.

WILSON AND BLAIR'S BISMUTH SULPHITE MEDIUM FOR THE ISOLATION OF *B. TYPHOSUS* AND *B. PARATYPHOSUS*

5 c.c. 20 per cent. glucose, 10 c.c. 20 per cent. anhydrous sodium sulphite and 5 c.c. standard bismuth (*vide infra*) solution are incorporated in 100 c.c. 3 per cent. nutrient agar. After boiling the medium for two minutes, 1 gram of exsiccated sodium phosphate and 1 c.c. 8 per cent. solution of ferrous sulphate crystals are added. The standard bismuth solution is made by mixing 60 grams bismuth citrate with 50 c.c. distilled water and then with 20 c.c. liq. ammoniac (sp. gr. 0·880) the volume being made up to 500 c.c. with distilled water.

(0·5 c.c. of 1 per cent. aqueous solution of brilliant green may be added to 100 c.c. of the medium.)

The use of this medium depends on the property of *B. typhosus* to reduce the sulphite to sulphide in the presence of glucose (the colonies becoming jet-black in colour), and the inhibition of *B. coli* by bismuth sulphite in the presence of an excess of sodium sulphite. (For further details, see W. J. Wilson and E. M. McV. Blair, 1931, *Journ. Hygiene*, vol. 31, p. 138.)

TELLURITE TRYPSIN COPPER SULPHATE SERUM-AGAR

(For the isolation of the diphtheria bacillus)

Several media containing compounds of tellurium have been devised for the isolation of *B. diphtheriae* from mixed cultures, and the following modification of Douglas' medium by Allison and Ayling¹ gives excellent results.

¹ V. D. Allison and T. H. Ayling, *J. Path. and Bact.*, 1929, vol. 32, p. 299.

Reagents required :

(a) 2 per cent. nutrient agar (pH 8) with a basis of either (1) Hartley's broth, or (2) meat extract + 1 per cent. peptone.¹

(b) Solution of copper sulphate (analytical reagent)² 10 per cent. in distilled water.

(c) Sterile tellurite trypsinised serum prepared as follows :

Horse serum	100 c.c.
Liquor Trypsin Co. ³	6 c.c.
Potassium tellurite ⁴ aqueous solution 2 per cent.	10 c.c.

In the preparation of the sterile tellurite trypsinised serum the serum is drawn from horse blood which has been allowed to clot. Liquor Trypsin Co. and a 2 per cent. solution of potassium tellurite are added in the proportions given above and allowed to stand at cold-room temperature for twenty-four hours. The mixture is then filtered through a Seitz bacterial filter into a sterile container, fitted with a siphon delivery tube and hooded pipette, and stored for use as required (*vide* diagram, p. 98); for small quantities the mixture may be distributed in amounts of 10 c.c. in sterile test-tubes and stored in the cold-room. It is important that the horse serum be used fresh and without the addition of chloroform.

To each 100 c.c. of nutrient agar, melted and cooled to 50° C., are added 10 c.c. of the sterile trypsinised serum tellurite mixture, and 0.5 c.c. of the 10 per cent. copper sulphate solution. After thorough mixing the medium is poured into sterile Petri dishes (about 14 c.c. in a plate of 4 in. diameter) and dried in the incubator. The medium is perfectly transparent and keeps well.

The advantage possessed by this medium is that it completely inhibits nose and throat organisms—such as *Staphylococcus*, *Streptococcus*, *D. catarrhalis*, pneumococcus, pneumobacillus, etc., while the diphtheria bacillus, Hofmann's bacillus and organisms of the diphtheroid group grow unrestricted, with typical colony appearances; moreover the spread of organisms of the *B. proteus* type, so often present in ear swabs, is inhibited.

B. diphtheriae grows typically as a round smooth colony with a dark centre well demarcated from a greyish white periphery; another type frequently met with is a tiny round greyish colony with a smooth glistening surface and no blackening. Colonies of Hofmann's bacillus grow as round glistening smooth colonies with a large grey or black central

¹ Parke, Davis & Co.

³ Allen & Hanbury.

² British Drug Houses, Ltd.

⁴ British Drug Houses, Ltd.

zone fading to a very thin grey periphery, and are lighter in colour than colonies of *B. diphtheriae*, from which they are readily distinguishable. Organisms of the diphtheroid group grow luxuriantly as large colonies, round, smooth and glistening, but their predominant characteristic is a dark brown coloration of the central zone as compared with colonies of the diphtheria bacillus. Other organisms which occasionally appear in cultures are: *B. proteus*, which grows as a large grey raised glistening colony showing little or no tendency to spread; yeasts, which grow as dull greyish-white colonies, and a Gram-positive tetracoccus, which appears as a dark brown rugose colony. These colonies are readily distinguishable from those of the diphtheria bacillus and the diphtheroid group. A low-power binocular magnifier (5-20 diameters) is of value in examining cultures.

Plates of the medium may be inoculated directly from swabs or from cultures on Löffler slopes, and it should be noted that at least twenty-four hours' incubation of the plates is, as a rule, necessary for the naked-eye recognition of colonies and the successful use of the medium.

McLEOD'S MEDIUM¹ FOR ISOLATION OF *B. DIPHTHERIAE*

This medium consists essentially of heated-blood-agar (chocolate agar) containing 0.04 per cent. of potassium tellurite. It differs from other diphtheria culture media in that the meat extract which it contains is never heated above 75° C. and is sterilised by filtration.

Add 1½ to 2 lbs. of minced meat to 1000 c.c. tap water at 48° C. and keep at this temperature for one hour. Squeeze out the juice through lint, leave this in the ice-chest overnight and filter through filter paper.

To 1000 c.c. filtrate add 20 grams peptone (Parke Davis & Co.) and 5 grams sodium chloride; warm at 45° C. until dissolved.

In order to adjust the reaction, take 50 c.c. and heat it to 80°-90° C. for fifteen minutes. Filter through paper. Determine the amount of N/10 NaOH required to bring 10 c.c. to pH 7.6 in the usual way (p. 92). Add to the bulk of the fluid an amount of alkali calculated on the basis of this titration.

Filter through a Seitz K clarifying film.

Refilter this filtrate through a Chamberland candle previously sterilised in the autoclave.

Distribute into flasks and tubes. One or two tubes should

¹ See J. Anderson, F. Happold, J. W. McLeod and J. Thomson, *J. Path. and Bact.*, 1931, vol. 34, p. 667.

be incubated for three days at 30° C. to control sterility. The remainder should be stored in the cold until required.

Mix equal parts of this broth and melted 5 per cent. agar in water.

Add 7–10 per cent. of freshly drawn defibrinated rabbit blood, and 0·04 per cent. of potassium tellurite.

Mix and heat at 75° C. for ten to fifteen minutes before pouring into Petri dishes.

HORGAN AND MARSHALL'S BLOOD TELLURITE MEDIUM FOR ISOLATION OF *B. DIPHTHERIAE*¹

Required:

(1) Ordinary nutrient agar, 2 per cent. at pH 7·6.

(2) Horse or ox blood (in 1 per cent. sodium citrate or 0·1 per cent. potassium oxalate).

(3) Potassium tellurite² 2 per cent. in distilled water.

It has been found preferable to substitute 0·5 per cent. glucose agar for ordinary nutrient agar.

To tubes of glucose agar (15 c.c.) melted and brought to 50° C. add 1·5 c.c. of both (2) and (3); mix and pour as plates. After pouring tilt the covers of the Petri dishes to dry off water of condensation. There is no risk of contamination.

Alternatively, the blood and potassium tellurite can be mixed in equal parts and stored in a refrigerator (not longer than three weeks); 3 c.c. of the mixture is added to the melted agar.

POPE AND SMITH'S MEDIUM FOR DIPHTHERIA TOXIN PRODUCTION

30 lbs. (13,600 grams) meat (minced and free from fat); 13 litres water.³

The meat and water are boiled in a steam cauldron and then filtered through a cloth filter bag.

The Infusion.—This is cooled to 30° C. and its pH adjusted to 8·0. Washed baker's yeast (100 grams) is now added and fermentation is allowed for six hours at 30° C.

The Meat.—After filtering off the infusion the meat is returned to the steam cauldron and 20 litres water added. The temperature is raised to 50° C. and kept at this for six

¹ E. S. Horgan and A. Marshall, *J. Hyg.*, 1932, vol. 32, p. 544. R. A. Q. O'Meara and H. J. Parish, *J. Path. and Bact.*, 1933, vol. 37, p. 165.

² British Drug Houses, Ltd.

³ The quantities specified refer to the preparation of this medium in large quantities for the manufacture of diphtheria toxin in serum laboratories.

hours. The reaction is adjusted to pH 8·0 with sodium carbonate and kept at 8·0 throughout digestion. Trypsin emulsion—preparation of Cole and Onslow (p. 83), but unfiltered—is added in 150 c.c. quantities every half-hour. After six hours' digestion the yeast-fermented extract is added to the digest in the cauldron together with 450 c.c. glacial acetic acid. The mixture is boiled for fifteen to thirty minutes and then filtered through a cloth bag.

The filtrate is adjusted to pH 8·0 and an addition of 0·3 per cent. maltose and 0·1 per cent. sodium lactate made. Sterilisation is carried out by aseptic filtration through a Seitz filter, followed by ten minutes' steaming at 100° C.

BORDET-GENGOU MEDIUM

(For the bacillus of Whooping-Cough)

Water	.	.	.	.	.	1000 c.c.
Glycerol	.	.	.	.	.	40 c.c.
Potatoes (minced or sliced)	.	.	.	.	.	500 grams

The mixture is well shaken and placed in the steam steriliser for one hour; it is then squeezed through a cheese-cloth and the fluid constitutes potato extract.

Make up an agar medium in the usual manner, as follows:—

Potato extract	.	.	.	.	.	500 c.c.
Salt solution (0·6 per cent.)	.	.	.	.	.	1000 c.c.
Agar powder	.	.	.	.	.	50 grams

The medium is distributed into tubes and sterilised in the steamer one hour daily for three successive days. For use, the melted medium is cooled to 45° C., defibrinated rabbit or human blood added in an equal amount, and the mixture allowed to solidify in the sloped position. Some workers use only 20 per cent. of blood, but the larger proportion is preferable when primary cultures are to be made.

Modified Method.

Potatoes are cleaned and cut into thin slices. 1 kilogram of the slices with 2 litres of distilled water and 80 c.c. glycerol are boiled until the potatoes are soft. The water lost in boiling is made up and the whole mixture filtered through muslin. The potato extract is then adjusted to pH 7·0. Make up 4 per cent. nutrient agar and to every 200 c.c. add 50 c.c. glycerol-potato extract; sterilise and keep as stock.

Make up N/2 solution of lactic acid (3·72 c.c. of lactic acid of sp. gr. 1·21 to 100 c.c. distilled water). Add to 200 c.c. agar while warm 4·5 c.c. of this lactic acid solution. Adjust pH to 5·9. When flask of agar has cooled to 45°–50° C. add 100 c.c. sterile defibrinated horse blood; shake and pour into Petri dishes.

DIEUDONNÉ'S MEDIUM

This is an alkaline blood medium used for the isolation of the cholera vibrio.

Equal parts of defibrinated ox blood and normal solution of caustic soda (40 grams per litre) are heated in the steam steriliser.

Volatile ammoniacal substances are formed in the blood-alkali mixture. These can be removed by repeated steaming.

Three parts of this mixture are added to seven parts of 3 per cent. agar.

The medium should be allowed to "ripen" for a day or two at room temperature, during which the pH reaches 9·9·6, which is the optimum reaction for the selectivity of the medium. At a lower pH other organisms grow, while at a higher the medium is too alkaline for the growth of the cholera vibrio. The "ripening" is due to carbon dioxide from the air, and loss of ammonia from the medium.

SMITH-NOGUCHI MEDIUM

This medium was introduced by Theobald Smith and developed by Noguchi, who used it with considerable success for the growth of pathogenic spirochaetes. It consists of a small piece of fresh sterile animal tissue, preferably rabbit kidney, covered with a long column of ascitic fluid on which is superimposed a layer of vaseline.

The medium is anaerobic, the function of the fresh tissue being to destroy by means of its catalase any hydrogen peroxide that might be formed by organisms, and be detrimental to their growth.

The essentials for success with this medium are: (a) good technique; (b) rigid asepsis in removing and manipulating the rabbit kidney; (c) suitable ascitic fluid: this must be clear, free from bile, and of high specific gravity; it must be sterile from the beginning, as sterilisation either by heat (57° C.) or by filtration may render it unsuitable.

Technique. Special long narrow tubes ($8 \times \frac{1}{2}$ in.) are employed. These should be cleaned, stoppered with cotton-wool and sterilised in the hot-air chamber. They are then wrapped up in batches of twenty-five in towels and autoclaved. Eight pairs of forceps and eight pairs of scissors are sterilised. It is advisable to keep these instruments solely for the purpose, and they are best sterilised in three large test-tubes $8 \times 2\frac{1}{2}$ in., plugged with wool, in the hot-air steriliser. One tube contains four pairs of scissors and four pairs of forceps, and the other two tubes each contain two pairs of scissors and two pairs of forceps.

A large healthy rabbit (1500–2000 grams in weight) is

selected, and fastened to a board. It is then bled by cardiac puncture (*vide* p. 107). It is essential that as much blood be removed as possible, so that the piece of kidney added to the medium is bloodless. The animal is now immersed completely in a bath of weak lysol (3 per cent.) for ten minutes, placed on the board and surrounded with towels soaked in weak lysol or 1 : 1000 perchloride of mercury solution, so that only the abdomen is exposed. For removing the kidneys the tube with the four pairs of scissors and forceps is used. The operation should now be done under an inoculating hood (p. 131). With a sterile knife and a pair of forceps make an incision the length of the abdomen and widely dissect away the skin. With another pair of scissors and forceps open the abdominal cavity and draw the intestines to one side. With fresh instruments remove the kidney from one side and place it in a sterile Petri dish. With the forceps pull over the intestines to the other side and with another set of instruments remove the other kidney in a similar manner to a second Petri dish. The animal should then be thoroughly examined for any intercurrent infection, such as snuffles, pneumonia, etc., and the kidneys should be retained only if the rabbit is perfectly sound.

The kidneys are cut up into small pieces, using a separate tube of instruments for each organ. The capsule is stripped and the fat at the hilum removed. Each kidney yields eight to eleven pieces, which are taken up with the second pair of forceps, and one piece is placed in each $8 \times \frac{1}{2}$ in. tube. Sterile ascitic fluid is run in by means of a 50 c.c. bulb pipette until the tubes are half full. Vaseline, previously sterilised by autoclaving in a 250 c.c. conical flask, is melted and added by means of a 10 c.c. pipette, forming a layer of about half-an-inch. The tubes are then incubated for forty-eight hours and examined for contamination.

The tubes are inoculated by first melting the vaseline and then introducing the inoculum to the bottom of the tube by means of a capillary pipette and rubber teat. Material is similarly withdrawn to be examined. Growth is evidenced by a clouding of the fluid at the bottom of the tube, which appears about the fourth to the tenth day of incubation. Turbidity, however, is not definite evidence of growth, as uninoculated control tubes also may show clouding just above the piece of tissue.

N.N.N. (NOVY, MACNEAL, NICOLLE) MEDIUM

For the growth of trypanosomes and *Leishmania*.

Meat extract is made in the ordinary way with rabbit or beef flesh, using 125 grams per litre of water. To this are added

peptone, 20 grams ; sodium chloride, 5 grams ; agar fibre, 20 grams ; and 10 c.c. normal sodium carbonate solution. After tubing, autoclaving and cooling to 50° C., there is added to the medium in each tube twice its volume of defibrinated rabbit blood (see p. 106). The contents of the tubes are mixed by rotation between the palms of the hands and allowed to solidify in the sloped position, preferably on ice. It is important to obtain a large amount of water of condensation.

Before inoculation the tube is placed in the upright position, and material is usually introduced by means of a capillary pipette. The growth of *Leishmania* occurs mostly in the water of condensation.

NÖLLER'S MODIFICATION OF N.N.N.

Agar	.	.	.	.	.	.	25 grams.
Glucose	.	.	.	.	.	.	20 grams.
Slightly alkaline bouillon	.	.	.	.	.	.	1000 c.c.

Prepare, filter and distribute into test-tubes in about 2 c.c. amounts. When required for use melt the medium, and when cooled to 55° C. add an equal or double volume of defibrinated horse blood, or undefibrinated rabbit blood.

LEPTOSPIRA MEDIA

These were introduced by Noguchi for the cultivation of the causal organism of Infectious Jaundice (Weil's disease). These media are rendered semi-solid by the addition of one-tenth of their volume of 2 per cent. nutrient agar.

- (1) Rabbit serum, 2 parts ; saline or Ringer's solution,¹ 6 parts ; citrated rabbit plasma,² 1 part ; neutral 2 per cent. agar, 1 part. The last is added fluid at 60° C. and the contents of the tubes are mixed by rotation to ensure a uniform distribution. A layer of sterile liquid paraffin $\frac{1}{2}$ -in. deep is then placed on the surface of the medium.
- (2) In a tube place 8 c.c. saline or Ringer's solution¹ at 55° C. Add 1 c.c. melted nutrient agar. Allow 20 drops

¹ Sodium chloride, 9 grams ; calcium chloride, 0.25 gram ; and potassium chloride, 0.42 gram, per litre.

² 10 c.c. of blood are allowed to drop from the ear vein of a rabbit (*vide* p. 106) into a tube containing 1 c.c. of 10 per cent. sterile sodium citrate solution, to prevent clotting. The blood is then centrifuged and the clear supernatant plasma pipetted off.

of blood from the ear vein of a rabbit to fall into the tube (*vide* p. 106). The tube is not shaken and the contents are allowed to become semi-solid without mixing. Sterility is tested by incubation at 37° C. for twenty-four hours. This medium is applicable when the organisms have been accustomed for some time to artificial cultivation.

SABOURAUD'S MEDIUM

A medium for the growth of fungi, consisting of

Peptone ("granulée de Chassaing")	. . .	1 per cent.
Maltose ("brute de Chanut")	. . .	4 "
Agar		1·8 "

which is made up as in the case of ordinary nutrient agar.

COOKED-MEAT MEDIUM

This is used for the cultivation of the anaerobes of the gas gangrene group. The original medium is known as "Robertson's bullock-heart medium," but the following modification of Martin and Lepper is recommended :

500 grams of fresh bullock's heart are minced, placed in 500 c.c. of boiling N/20 caustic soda and allowed to simmer for twenty minutes, by the end of which time the neutralisation of the lactic acid will be ensured and the pH of the liquor should be about 7·5. The liquid is drained off through a muslin filter and, whilst still hot, the minced meat is pressed in a cloth and allowed to dry partially by being spread on a cloth or filter-paper. In this condition it can be introduced into test-tubes without soiling them. Enough should be placed in each to occupy about 5 cm. of the tube, and 1 per cent. infusion broth, adjusted to pH 7·5, added until the liquid stands 1 cm. or more above the meat. The tubes must be kept in a bath of boiling water for half-an-hour to drive off any dissolved oxygen. They are then autoclaved at 120° C. for twenty minutes. The inoculum should be introduced towards the bottom of the tube in contact with the meat.

It is usual to cover the surface of the medium with a layer of sterile liquid paraffin, $\frac{1}{2}$ in. deep, although this is not necessary.

It is convenient also to distribute cooked-meat medium in 10 c.c. amounts in small screw-capped bottles, without adding liquid paraffin. This method is convenient for anaerobic cultures, and also for the preservation of stock cultures of aerobic organisms.

ALKALINE EGG MEDIUM

Beat up the yolk of one egg and the whites of two, adding 6 c.c. of N/1 caustic soda. Transfer this to a large flask and make up to 500 c.c. with tap water. Heat slowly to 95° C. for 1½ hours, then filter through cotton-wool and distribute into tubes. Cover the medium with a layer of liquid paraffin and autoclave. A clearer medium is obtained by using the whites of two eggs without any yolk, adding 4 c.c. of normal caustic soda, and making up to 330 c.c. with tap water.

BOECK AND DRBOHLAV'S MEDIUM FOR THE CULTIVATION OF AMOEBAE

Four fresh eggs are well washed with soap and water, wiped over with alcohol, and broken into a sterile bottle containing glass beads. 50 c.c. Locke's solution¹ are then added and the mixture thoroughly shaken. Test-tubes are filled with sufficient of the medium to form short slopes of 1-1½ in., and the slopes are coagulated in the inspissator at 70° C. They are then sterilised by autoclaving. To each tube is added a mixture of 8 parts sterile Locke's solution and 1 part of sterile human serum inactivated at 55° C. for half-an-hour. The fluid should cover the solid medium to a depth of half-an-inch, and the tubes are then incubated to test for sterility. Instead of the serum mixture a 1 per cent. solution of crystallised egg albumen in Locke's solution, sterilised by filtration through a Seitz filter, may be substituted. The initial reaction of the medium, which varies between pH 7·2-7·8, does not require adjustment.

Dobell and Laidlaw have modified the medium in the following manner:—

Sterile horse serum is inspissated in the sloped position for one hour at 80° C. It is then covered with serum or egg-albumin solution as above. A small quantity of solid rice starch (sterilised in thin layers at 180° C. for one hour in the hot-air oven) is added. Amoebae grow luxuriantly in this medium, and the whole life cycle, including encystment, may be observed.

WHEY MEDIA

These media are useful for the growth of *B. acidophilus* (p. 357).

Whey Broth.—Add 10 per cent. hydrochloric acid to skimmed milk heated to 80°-90° C., in amount just sufficient to precipitate

¹ Sodium chloride, 9 grams; potassium chloride, 0·42 grams; calcium chloride, 0·24 grams; sodium bicarbonate, 0·1-0·3 grams; water, 1 litre.

the casein. Filter through cotton-wool and adjust the pH to 6·8–7·0. Now add 0·5 per cent. peptone, autoclave at 15 lbs. pressure for fifteen minutes, and filter.

Whey Agar.—Made from whey broth by the addition of 1·5 per cent. agar.

USE OF CULTURE MEDIA

Only general methods are described here. Special methods applicable for particular purposes are referred to in the appropriate sections—*e.g.* under special media.

STORAGE AND DISTRIBUTION OF CULTURE MEDIA

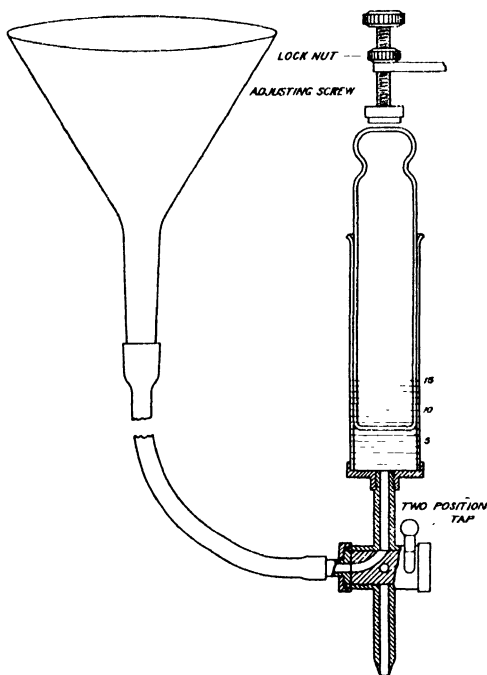
Culture medium after being made is either stored in bulk, or distributed in tubes, small bottles, or Petri dishes (*vide infra*). It is convenient to store fluid media in 500 c.c. and 250 c.c. amounts in 20 oz. or 12 oz. screw-capped bottles. The bottles are sterilised with the caps tightly screwed on, so that the medium remains sterile and without evaporation. For solid media, storage in 250 c.c. and 100 c.c. amounts in 12 oz. and 6 oz. screw-capped bottles is recommended. The 250 c.c. amounts are useful for subsequent distribution into tubes or small bottles. The 100 c.c. amounts are extremely convenient for pouring into Petri dishes—*e.g.* nutrient agar, MacConkey's medium—or when melted and cooled to 55° C. the addition of 10 c.c. defibrinated horse blood or serum will make sufficient blood-agar or serum-agar for seven plate cultures (in 4 in. Petri dishes) (*vide p.* 127).

USE OF SOLID MEDIA

For immediate use the medium is allowed to solidify in sterile stoppered test-tubes either by cooling after having been melted by heat, as in the case of agar or gelatin (*vide p.* 84), or by coagulation in an inspissator, as in the case of solidified serum or egg media (*vide p.* 103). The tubes are plugged with cotton-wool, and sterilised in the hot-air oven before the addition of the medium.

Alternatively 1 oz. screw-capped bottles (p. 126) can be substituted for test-tubes.

Tubing of medium is conveniently carried out by means of a sterile 6 in. glass funnel (fixed in a burette stand) with a short length of rubber tubing and glass delivery nozzle fitted to the stem and controlled by a pinchcock. During the tubing the funnel



is covered with the lid of a large sterile Petri dish to avoid aerial contamination.

The latest improved model of automatic filler devised by T. H. Ayling can be recommended for tubing media.¹ It consists of a glass funnel 7 inches in diameter, connected by rubber tubing to a metal 3-way stopcock which in turn is connected to

¹ Supplied by R. B. Turner & Co., London.

an all-glass syringe of 15 c.c. capacity. The syringe is of the three-piece type, but without the nozzle, and the plunger is hollow, as the head of liquid will not lift a solid glass piston. The barrel is graduated to 15 c.c. by 0.5 c.c., and the numbers are engraved so as to be readable when the syringe is vertical. The syringe is connected to the stopcock by means of a metal screw fitting. A clamp on a swivel mount secures the lower end of the syringe. The amount of fluid delivered is determined by the adjustable screw. A very thin film of liquid paraffin well rubbed on the surface of the plunger and barrel will make for smoother working. The action of the filler is simple. The head of medium in the funnel forces up the plunger until it is stopped by the adjustable screw. The handle of the stopcock is then turned and the syringe empties itself under the weight of the plunger. Air bubbles in the syringe are removed by first filling the apparatus and then turning the syringe by means of the swivel mount. This brings the nozzle of the syringe uppermost, and when the plunger is pressed home the air passes through the rubber tube and liquid in the funnel. If a smoothly working syringe is used, very little head of pressure is necessary, and the height need not be greater than 18 inches.

Once set, the accuracy of the filler is much greater than that of an ordinary pipette, while media can be tubed with greater rapidity. It works equally well with melted agar or gelatin, provided that fresh hot supplies are available, and the syringe and stopcock are washed out immediately after use.

When tubing agar or broth with the filler, the medium is run into clean but not sterilised test-tubes. These are then plugged with cotton-wool and sterilised in the steamer or in the autoclave as indicated under the description of the various media.

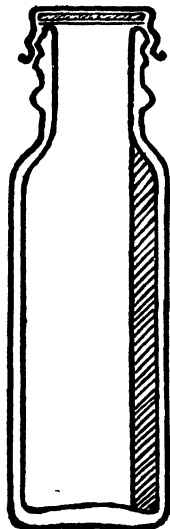
Alternatively the agar or broth is distributed into small bottles (p. 126), the cap is screwed tightly on, and the whole container suitably sterilised.

Depending on the method of inoculation to be used, solid media are solidified in tubes as follows :—

- (a) *Upright, for "puncture" or "stab" culture.*—The test-tube or small screw-capped bottle is half filled with the medium (about 12 c.c.) which is allowed to solidify in the upright position. It is inoculated by plunging a long straight wire (*vide* p. 128), charged with the material, vertically down the centre of the tube. This method is used for anaerobic

cultures in glucose agar, and for testing the liquefaction of gelatin.

- (h) *Sloped, for "stroke" culture.*—This is often called a "*slope*" or "*slant*," and ensures a maximum surface of the medium exposed to the air. Quantities of 5 c.c. of medium for an ordinary $6 \times \frac{3}{8}$ in. tube are sufficient. When a large number of agar tubes have to be sloped, special trays, which allow the tubes to be laid at the correct angle, are useful, and moreover they can be stacked one upon another so that very little bench space is required during solidification. Fresh agar slopes after cooling contain "water of condensation" at the foot of the tube, and the tubes should be stored and handled in the vertical position to prevent the fluid from flowing over the surface of the medium or entering the cotton-wool stopper.



- 1 oz. screw-capped bottles¹ can conveniently be substituted for test-tubes. The aluminium cap should have a waxed millboard washer. 5 c.c. amounts of the medium are added to the bottles and the cap tightly screwed on. Instead of being sloped, the bottles are merely placed in the horizontal position during solidification (on the bench for agar, and in the inspissator for Löffler's medium and the various egg media, *q.v.*). The position of the medium is shown in the figure, the amount of 5 c.c. being just sufficient to reach the top of the shoulder.

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¹ These bottles, type H53, are supplied by the United Glass Bottle Manufacturers Ltd. See footnote, p. 79.

A larger surface for the same amount of medium is obtained in these bottles than in the $6 \times \frac{5}{8}$ in. test-tubes. Being tightly sealed there is no evaporation and the surface of the medium is always moist.

Plates.—Where a large surface is necessary, as in the separation of organisms from mixtures (*vide p. 132*), the medium—*e.g.* agar or gelatin—is allowed to solidify in the form of a thin layer in a Petri dish. For a dish of 4 in. diameter, 14 c.c. of medium are ample. The *melted* medium is poured into the dish with the necessary precautions to avoid contamination. Medium which has been tubed—*e.g. vide supra* (a)—can be melted and used for pouring plates.

In separating organisms in mixed cultures by spreading the material in Petri dishes, it is essential that the surface of the medium should be dry. When plates have been poured, the steam from the hot liquid condenses on the surface of the medium and



this moisture is undesirable for cultural work. It is removed by drying the poured plates in the incubator at 37° C. for one hour. The lid of the dish is first laid in the incubator (see diagram); the portion containing the medium is then inverted (so that the surface of the medium is downwards) and placed in the incubator so that the free edge rests on the lid. If care is taken to avoid disturbing dust, there is very little risk of contamination of the medium by air organisms.

Shake Cultures.—Agar or gelatin medium in tubes—*e.g.* (a), *vide supra*—is inoculated in the melted condition at a temperature which keeps the medium fluid, but is not *immediately* lethal to the organisms inoculated (*e.g.* 45° – 50° C.). The contents of the tubes are mixed by rotation between the palms of the hands and then

poured at once into a Petri dish, as in water examination (*vide* p. 233), or left to solidify in the tube so that colonies may develop in the depth of the medium, as when separating anaerobes. In the latter case the test-tube is filed and broken, and the colonies "picked out" of the medium exposed in this way.

USE OF FLUID MEDIA

Fluid media are used in (1) test-tubes stoppered with cotton-wool, the tubes being about half filled; or (2) 1 oz. screw-capped bottles, in 5 or 10 c.c. amounts—broth and peptone water; and in 5 c.c. amounts or less—fermentation media; or (3) stoppered flasks, according to the quantity of medium required for individual cultures; or (4) in 100 c.c. amounts in 6 oz. screw-capped bottles for blood culture (*vide* p. 142).

INOCULATION OF CULTURE MEDIA

According to the nature of the medium and the inoculum, various methods are employed for inoculation, and the following instruments are commonly used:—

"PLATINUM LOOP."—This consists of a piece of platinum wire, No. 23 S.W.G., $2\frac{1}{2}$ in. long, with one end fused into a glass rod, or inserted into a special aluminium holder. The other end of the wire is bent in the form of a loop, care being taken that the loop is flat and completely closed. Owing to the high cost of platinum, "resistance" wire No. 25 S.W.G. may be used as an efficient substitute.

The wire is sterilised by holding it vertically in a Bunsen flame so that the whole length becomes red-hot at the same time. The platinum loop is the most useful of all the inoculating wires. Not only can a considerable amount of solid culture be taken up by it, but also a large drop of fluid.

STRAIGHT WIRE.—This is similar to the foregoing, but without the loop. It is used for stab cultures, and also for picking off single colonies.

LONG STRAIGHT WIRE.—A wire $4\frac{1}{2}$ in. long mounted on a holder. It is employed for deep-stab inoculation when working with anaerobes.

THICK WIRE, particularly with a loop, is very useful on account of its rigidity for lifting thick viscid sputum and tenacious growths.

WIRE WITH LANCE-HEAD.—This is made with thick wire (*e.g.* resistance wire), one end being flattened out and filed to a lance-head or diamond shape. It is employed for making scrapings from organs, for picking off tough colonies and for disintegrating felted cultures—*e.g.* fungi.

SCALPEL.—This instrument, sterilised by dipping in alcohol and flaming, is used for making inoculations with scrapings from tissues and ulcers, etc.

STERILE CAPILLARY PIPETTES.—These are made by heating the middle of a piece of quill tubing, 5 mm. bore and 5 in. long, and when melted pulling out the two halves, thus forming two pipettes. The capillary ends, which should not be too thin, are sealed in the flame, and the other ends are plugged with cotton-wool. They are placed in a large test-tube $15 \times 2\frac{1}{2}$ in., which is then stoppered with cotton-wool and sterilised by dry heat. Before use, the tip of the capillary portion is broken off and a rubber teat fitted to the other end. These pipettes are necessary for inoculating Smith-Noguchi medium and cooked-meat medium (*q.v.*), and are very useful in many bacteriological manipulations.

STERILE PIPETTES (10–100 c.c.) are used when large amounts of fluid inoculum have to be added to a medium.

Graduated pipettes are employed when measured quantities of material are used for inoculation (*vide* water examination).

✓ TECHNIQUE OF INOCULATING TUBES

The following routine methods are employed by the writers and are recommended to beginners:—

Inoculation of one "slope" from another.—The two

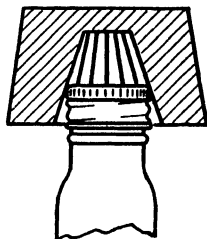
tubes are firmly held at their lower ends between the thumb and first two fingers of the left hand, with the sloped surface of the medium towards the worker. The tube containing the growth should be on the left and the uninoculated tube on the right. With the right hand loosen the cotton-wool stoppers by rotating them in the mouths of the tubes so that they may be removed easily. Take the holder of the inoculating wire at its end between the thumb and first two fingers of the right hand (as in holding a pen). 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 third finger of the right hand, and flame the mouth of the tube. Pass the needle into the tube and touch a portion of the medium free from growth to ascertain if the needle is sufficiently cool. If too hot, the wire will melt the agar, causing a furrow, and might of course kill the organisms in removing the growth. When the wire is cool, the growth is scraped from the surface, care being taken not to wound the agar. Withdraw the wire, remove the stopper from the other tube with the crooked little finger and flame the mouth of the tube. Insert the wire charged with the growth and lightly smear the surface of the agar. Withdraw the wire and sterilise it, flame the mouths of the tubes and replace the stoppers. The nature of the inoculated material and also the date should be written on the tube by means of a grease pencil, or on a gummed label which is then affixed to the tube.

With *stab cultures*, the tubes are held similarly and the straight wire charged with bacterial growth is plunged into the centre of the medium, care being taken to withdraw the wire in the same line, and not to cause splitting of the medium.

In *inoculating a fluid medium*, such as broth, from a solid culture, the tube should be inclined almost to the horizontal and the growth on the loop deposited on the wall of the tube just above the surface of the liquid at the lower end of the tube. On returning

the tube to the vertical position the inoculum is below the surface of the broth.

Screw-capped Bottles.—When inoculating the small screw-capped bottles which are used as substitutes for test-tubes, essentially the same procedure is carried out as above. Before the bottles are held in the hand it is advisable to loosen the screw-cap, as this is usually tightly screwed before sterilisation to seal the bottle effectively. If the caps are very tight they can easily be loosened by means of a bored-out rubber bung, a section of which is shown in the sketch. A bung about two inches across is suitable, and by means of a cork-borer a number of holes are bored in a slanting direction round a diameter of $1\frac{1}{4}$ inches, so that the whole centre is removed, leaving a conical-shaped cavity. It is preferable to have the wall ridged, as it grips the cap more easily. The bung can be held in the hand, or suitably mounted just below the edge of the bench. The bottles are held exactly as test-tubes, and the cap is held in the same way as the cotton-wool stopper. The bottles are then unscrewed from the cap, the wire is introduced and the inoculation made. The cap is now loosely screwed on, and when the bottle is ready for the incubator the screw-cap is tightened.



INOCULATING HOOD.—It is advisable, as far as possible, to carry out certain inoculation procedures under a hood in order to minimise the chances of aerial contamination.

The hood employed by us has a wooden top and back, and two side windows. This is placed on the laboratory bench so that a chamber is formed, open only at the front. The top overhangs the bench by about 18 inches. The front is then closed by curtains made of calico, or waterproof material such as jaconet, and the operator works completely under the hood. Ventilation is secured by two holes in the roof, covered with fine-mesh wire gauze. The table under the hood is covered by a towel soaked in 1 : 1000 perchloride of mercury solution, so that any organisms deposited in dust are destroyed. The advantage of the hood depends on the relative absence of dust and air currents, which are liable to produce contamination of medium, etc., exposed in the process of inoculation. We have used the inoculating hood with considerable success in the preparation of blood-agar plates and other highly nutritive media, and in conducting autopsies on animals under aseptic conditions.

The bench on which the hood is fitted should have a gas supply for the Bunsen burner. The hood may be lighted by means of an electric lamp suspended from the top.

SEPARATION OF MIXED CULTURES

1. BY PLATING.—The term “plating” is generally applied to the inoculation of medium in Petri dishes, usually by successive strokes or spreading. The platinum loop is charged with the bacterial mixture, pus, fragment of tissue, etc., and several strokes in series are made on the surface of the medium (either in a Petri dish or sloped in tubes) without recharging the wire (*vide* p. 332).

An alternative method for Petri dishes is to employ a spreader. This is made by bending a piece of glass rod 3 mm. diameter at a right angle in the blowpipe flame, the short limb, used for spreading, being 1 in. long. A small amount of the bacterial mixture is placed on the plate with the inoculating loop or capillary pipette. By means of the spreader, previously sterilised by boiling and then cooled, the material is evenly distributed over the surface. The spreader is then transferred to a second plate, which is similarly inoculated. Thus the medium in the second dish is inoculated merely with the organisms carried over by the spreader from the first.

By these methods the bacteria are gradually wiped off the wire or spreader so that they are ultimately deposited singly. Generally from each bacterium an isolated colony will grow; a single colony may be subcultured on fresh media and so yield a pure growth. *In order to ensure separation, the surface of the medium must be dry.*

2. BY PLATING DECIMAL DILUTIONS OF THE INOCULUM.—A series of tubes of melted agar or gelatin are inoculated with successive decimal dilutions of the infected material and then the medium in each tube is poured into a Petri dish and allowed to solidify. By dilution, the bacteria are separated from one another, and on incubation the resulting colonies

are distributed singly throughout the solid media. This method is used when the number of viable organisms present in a fluid has to be ascertained, as in water and milk examinations, and in blood in cases of septicaemia.

3. BY HEATING AND SUBSEQUENT PLATING.—This method is employed where the organisms to be obtained in pure culture are more resistant to heat than the remainder of the bacteria present. This method applies especially to spore-bearing organisms, such as the anaerobes (p. 388), the spores of which survive the heating. The mixture of bacteria is heated to 80° C. for half-an-hour and then plated. The spores form individual colonies, which may then be “picked off.”

Plate cultures should have the nature of the material, and also the date, written on the glass of the Petri dish by means of a grease pencil. Agar plates are incubated in the inverted position—*i.e.* the lid of the plate is underneath and the grease-pencil writing should be on the portion of the dish containing the medium. On the other hand, gelatin, because it is liquefied by many organisms, is incubated with the lid uppermost, on which the necessary pencil notes are made.

Care must be taken in *picking off single colonies*, particularly when they are very close to one another, that the point of the needle does not touch any of the neighbouring colonies. The plate should first be looked at through the medium by holding it up to the light. The lid should be removed and the dish held round the side by the thumb and middle finger of the left hand. The colonies selected should be marked by grease-pencil rings on the bottom of the dish. To pick off the colony, first sit down with both elbows on the bench. Hold the plate vertically with the left hand, then grasp the holder of the wire like a pen, with the fingers quite close to the wire. Steady the right hand by placing the little finger on the

left thumb in the way artists support the hand when painting. The selected colony is then easily removed without touching the others. Lay the plate on the bench, withdraw the right hand to the other end of the holder and inoculate the required medium in the manner previously described.

Plate Culture Microscope

There are on the market low-power binocular magnifiers which are extremely useful for examining plate cultures of organisms; they have a long working distance so that a colony can be "picked off" the plate while using the instrument. When dealing with bacteria forming small delicate colonies, or where the colonies of the desired organism are few in number, the low-power binocular is invaluable. A magnification of ten diameters is useful for general work, but by interchangeable eye-pieces and objectives, magnifications from three to thirty diameters are available.

4. BY SHAKE CULTURE IN TUBES (*vide* p. 127). This method is sometimes used in the separation of anaerobic organisms.

5. BY THE USE OF SELECTIVE MEDIA.—Such media as MacConkey's for the coli-typhoid group, Dicudonné's for the cholera vibrio, the various tellurite media for *B. diphtheriae*, etc., have been devised so that the majority of the organisms other than those for which the media are used will not grow, and pure cultures can thus be obtained.

6. BY ANIMAL INOCULATION.—Advantage is taken of the fact that laboratory animals are highly susceptible to certain organisms—for example, the mouse to the pneumococcus. If a mixture of organisms containing pneumococci—*e.g.* sputum—be inoculated subcutaneously into a mouse, the animal dies of pneumococcal septicaemia in twenty-four to thirty-six hours, and from the heart blood the organism can be obtained in pure culture. Similarly the tubercle bacillus can be isolated from contaminating organisms by inoculation of a guinea-pig. The tubercle bacilli are found in pure culture in the resulting lesions.

INCUBATION

Students and others commencing work in a laboratory should familiarise themselves with the mechanism of the incubator whereby any desired temperature may be constantly maintained. Incubators may be heated by electricity, gas or oil, according to the facilities of the laboratory.

All bacteriological laboratories have one or more incubators working at 37° C. This temperature, which is the optimum for practically all pathogenic organisms, is that referred to when speaking of incubation without mentioning the temperature.

Some laboratories have a warm room heated by gas or electricity, and kept at 37° C., in which large quantities of material can be incubated. The warm room should have double doors with a space between them to act as an air-lock. To enter the room the outer door is opened, the air-lock entered, and the outer door closed. The second or inner door which leads into the room is now opened, the hot room entered, and the inner door closed. This method of entering prevents cold air from the outside lowering the temperature. When leaving the hot room the inner door is closed before the outer door is opened. The hot room has a regulating mechanism similar to the ordinary incubator to keep the temperature constant, and if electrically heated it should be fitted with a device to cut off the current for the room at the main switch, if the temperature rises above 40° C.

Other temperatures for incubation are 30° C., used for cultivating leptospiras, and 22° C. ("cool incubator"), used for certain fungi and for gelatin cultures.

Gelatin medium melts at about 24° C.

In order to prevent drying of the medium when prolonged incubation is necessary, as in the cultivation of the tubercle bacillus, the mouths of the culture tubes are sealed with paraffin wax, or covered with special rubber caps. Under these circumstances, however, we strongly advise that small screw-capped bottles (p. 126) should be used instead of test-tubes.

METHODS OF ANAEROBIC CULTURE

Anaerobes are usually defined as organisms that will grow only in the absence of free oxygen (see p. 15). It has been shown that it is not free oxygen by itself which is inimical to the growth of these organisms, but that when molecular oxygen is present, a peroxide is formed, probably hydrogen peroxide, which prevents their multiplication. Anaerobes may be cultivated, therefore, either by preventing the admission of oxygen to cultures, or by destroying the peroxide as fast as it is formed, by means of catalase derived from fresh animal or vegetable tissue.

In the *Smith-Noguchi* method, for example, a combination of these methods is used. The cultures are sealed from the air by a vaseline plug; and any peroxide that may be formed is at once destroyed by the catalase present in a piece of fresh sterile rabbit kidney. This method is described on p. 118.

The method usually employed to establish anaerobic conditions is to remove the oxygen from the atmosphere surrounding the culture, the oxygen being sometimes replaced by an inert gas.

The simplest method of securing anaerobiosis is by growing the organisms in solid media. Deep agar tubes are convenient and efficient for the purpose. The addition of 1 per cent. glucose to the medium is of value, particularly when cultivating the saccharolytic group of anaerobes. Glucose acts as a reducing agent, thereby removing oxygen from the medium, and further serves as a suitable pabulum for bacterial growth. The agar may be inoculated when solid by means of a long straight wire (*vide* p. 129). The colonies develop best in the depth of the tube, becoming fewer and smaller towards the surface. No growth is usually noted in the top half-inch of the medium. An alternative method is to melt the agar, cool it to 50° C. and introduce the inoculum by means of a capillary pipette. The contents of the tube are mixed by rotation between the palms of the hands.

The agar is then rapidly solidified by placing the tube in cold water. The colonies develop in the deep portions of the tube, usually separated from one another.

Glucose-broth can easily be rendered completely anaerobic. Long tubes, $8 \times \frac{1}{2}$ in. (Noguchi tubes), are half filled with the medium and are placed in the steamer for half-an-hour or in boiling water for five minutes. Sterile melted vaseline is then poured on the surface of the medium and the tubes are rapidly cooled. The glucose and the heating remove all oxygen, and the vaseline effectively seals the medium from the air. Inoculation is made by means of a capillary pipette after melting the vaseline. Gas-producing anaerobes should not be cultivated in this medium, as the gas formed will force out the vaseline seal.

Robertson's cooked-meat medium (p. 121) is also very useful for anaerobic work. The sterilised tissue contains reducing substances, which are effective in maintaining anaerobic conditions at the bottom of the tube. The reducing activity of the meat is shown by the pink colour in the lower layers due to the reduction of haematin.

REMOVAL OF OXYGEN BY MEANS OF PYROGALLIC ACID AND CAUSTIC SODA

When pyrogalllic acid and caustic soda are mixed together, the mixture rapidly absorbs oxygen and becomes dark brown in colour. Many types of apparatus have been devised for the cultivation of anaerobes on plates, in which this method is used for absorbing oxygen. They are, however, inconvenient, and are not specially recommended.

For media in ordinary test-tubes, **BUCHNER'S METHOD** is applicable. The test-tube containing the medium is placed in a Buchner's tube, a stout-walled tube $8\frac{1}{2} \times 1$ in., with the lower end constricted so that the test-tube placed therein does not reach to the bottom of the tube. The tube is furnished with a well-fitting rubber bung. Some solid pyrogalllic acid is placed in the bottom of the tube, strong caustic soda is added, the inoculated tube is quickly introduced, and the rubber bung immediately inserted. The oxygen is rapidly absorbed and fairly satisfactory anaerobic conditions are obtained.

McINTOSH AND FILDES' JAR

This apparatus is easy to manipulate, and the degree of anaerobiosis is easily observed by means of the methylene-blue indicator inside the jar.

The principle of the apparatus is that spongy palladium or spongy platinum acting as a catalytic agent causes the slow combination of hydrogen and oxygen to form water. The jar itself (8×5 in.) is made of stout glass or of metal, and has a tight-fitting lid that can be clamped down. The lid is furnished with a tube and tap, so that hydrogen may be introduced into the jar. Suspended from the lid by means of two wires, which are connected to terminals, is a small glass or porcelain spool around which is the spongy palladium. This spongy palladium is made by immersing asbestos in a solution of palladium chloride and allowing it to dry; on heating in the blowpipe, the palladium is deposited in a black amorphous spongy layer on the asbestos. A fine coil of resistance wire is wound round the palladinised asbestos and the ends are connected to the two wires supporting the spool, so that an electric current can be passed through and the spongy palladium heated. The spool is surrounded by wire gauze, which, on the principle of the Davy lamp, prevents an explosion of the hydrogen and oxygen mixture. The amount of current passed through depends on the thickness of the resistance wire and on the voltage of the electric supply. The current passed by three carbon filament lamps in parallel (two 16 c.p., and one 8 c.p.) is suitable with the ordinary domestic supply.

Petri dishes or tubes are placed inside the jar, and also an indicator to show that anaerobiosis is maintained. This consists of a mixture in a test-tube of equal volumes of (a) $N/10$ 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; the mixture is boiled until the colour is 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.

The lid is clamped down and the jar connected to a hydrogen supply. The current is turned on so that the palladinised asbestos may be heated. The combination of oxygen and hydrogen takes place quietly in the jar. Water is formed, and more hydrogen enters to take the place of the oxygen consumed. After about twenty minutes all the oxygen is used up, and the tap is then turned off and the hydrogen supply disconnected. The jar is placed in the incubator, and within a few hours the indicator tube containing the methylene blue should be colourless, showing that complete anaerobiosis is established.

In spite of the wire gauze round the palladinised asbestos, explosions sometimes occur, and it is advisable always to place the anaerobic jar, if made of glass, inside a box while the current is passing through the spool.

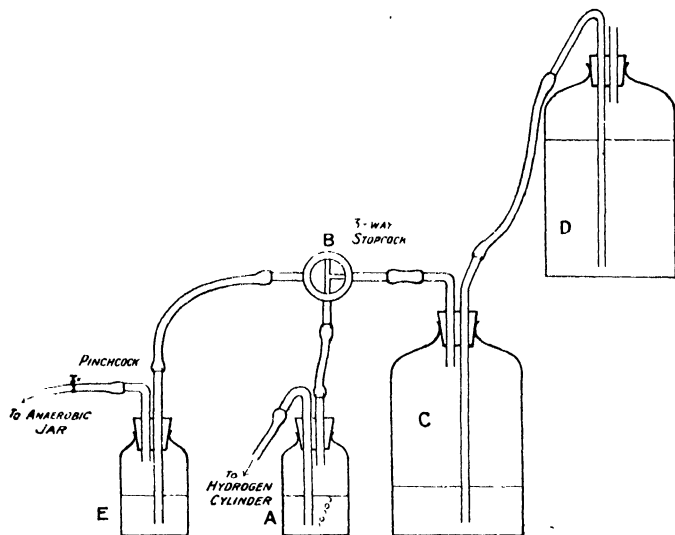
HYDROGEN SUPPLY

A hydrogen supply may be obtained from a Kipp's apparatus by the action of sulphuric acid on zinc. The gas must be purified by passing through three wash-bottles : (1) containing a ten per cent. solution of lead acetate, to remove sulphuretted hydrogen ; (2) containing a ten per cent. solution of silver nitrate, to absorb arseniuretted hydrogen ; and (3) containing a mixture of pyrogallic acid and caustic soda, to remove oxygen.

It is more convenient, however, to obtain hydrogen from a cylinder containing the compressed gas. The commercial hydrogen so obtained is suitable for use in the various anaerobic apparatus employed. The hydrogen cylinder cannot be connected directly to the McIntosh and Fildes' jar, as the pressure is too great. By the following apparatus, however, the amount of hydrogen entering the jar can be measured, and its pressure controlled.

Hydrogen from the cylinder is passed through the wash-bottle A, the rate of flow being estimated by the bubbles of gas rising through the water. From this the gas passes through the three-way stopcock B to a large jar C filled with water. This jar should have a capacity of 15-20 litres, and may be

graduated in litres by adding measured amounts of water and marking the levels on a strip of $\frac{1}{2}$ in. adhesive plaster attached to the jar. The jar is furnished with a rubber stopper and glass tubing as illustrated. The hydrogen entering C forces the water into a similar jar D, which is placed on a support four feet above C. When sufficient hydrogen has entered C, the cylinder supply is turned off. The stopcock is then turned so



that the gas can pass from C through the wash-bottle E, where the hydrogen flow can be estimated. As the gas escapes from C, it is replaced by water siphoned over from D. The pressure of the hydrogen passing through E depends only on the height of the jar D above C.

Care must be taken to make the rubber stoppers in the jars and wash-bottles air-tight. This can be done by means of sealing-wax. Thick-walled "pressure" rubber tubing must be employed for all the connections.

Alternatively, the hydrogen cylinder may be connected directly to the anaerobic jar if fitted with a reducing valve to supply at a pressure not exceeding 250 mm. of mercury, such as an "Endurance" gas regulator. It is essential to have a U-shaped mercury manometer working up to 300 mm. to show either the positive pressure (when gas is passing from the cylinder), or negative pressure when exhausting the jar, or

when the pressure falls owing to the oxidation of the hydrogen. The manometer is connected to the tube between cylinder and anaerobic jar by means of a glass T-piece.

BLOOD CULTURE

Requisites :

- (1) A 10 c.c. Record syringe or an "all-glass" syringe (with a firmly fitting needle) sterilised by boiling in water for 10–15 minutes; the syringe must not come into contact with any antiseptic; it should not be removed from the steriliser until it is immediately required, and the parts should be taken out of the steriliser and fitted together with the aid of forceps so that the needle, nozzle and piston are not touched by the fingers. Alternatively the syringe, if "all-glass," may be sterilised beforehand in the hot-air oven as described on p. 181, but this procedure is not recommended for the Record type, as the cement or solder is apt to melt.
- (2) Gauze or cotton-wool, bandage, antiseptic (4 per cent. iodine in spirit, or 1 : 20 carbolic acid), methylated spirit, collodion, dissecting forceps, Bunsen burner or spirit lamp.
- (3) 100 c.c. sterile Hartley's Broth in a stoppered flask, or preferably a special blood-culture bottle, described later. (0·2 per cent. sodium citrate may be incorporated in the medium to prevent coagulation of the blood.)

The blood is drawn by vein puncture. The skin of the patient's arm at the bend of the elbow is *thoroughly sterilised*, particularly to obviate contamination of the culture with skin organisms—c.g. *Staphylococcus albus*. Several turns of a bandage are applied round the upper arm about the middle of the biceps to render the veins turgid, or a piece of rubber tubing firmly, but not too tightly, wound once round the arm and clipped with pressure forceps provides a convenient and easily

released tourniquet for the purpose. The turgescence of the veins can be increased by the patient's alternately opening and clenching the hand. The needle of the syringe is inserted into a prominent vein and 5-10 c.c. of blood are drawn into the syringe. The tourniquet is then released. The needle is now withdrawn from the vein and detached from the syringe by means of forceps so that the nozzle is not touched by the fingers. The flask of broth is unstoppered and the mouth of the flask flamed. The blood is added to the broth and the flask re-stoppered. The blood and broth are thoroughly mixed by rotation of the flask. The flask is incubated at 37° C.

The syringe and needle should be washed out at once with cold water. The puncture wound is dressed with gauze or cotton-wool, and collodion.

When the flask has to be transported some distance to the laboratory it is advisable to stopper it with a sterile rubber cork sterilised by boiling, and inserted into the flask with flamed forceps.

In suspected cases of enteric fever, instead of broth as the culture medium, sterilised ox bile or 0.5 per cent. sodium taurocholate broth may be used. 5 c.c. of blood are added to 10 c.c. of bile or to 50 c.c. of taurocholate solution.

Blood-culture Bottle.—Instead of using a flask of broth with a cotton-wool or rubber stopper, as described above, the following container is much more simple and convenient, especially where the patient is some distance from the laboratory.

It consists of a 6 oz. (actually 170 c.c.) white "medical flat" bottle, with a screw cap, as used for storing nutrient agar, etc., in 100 c.c. amounts (see p. 79). A small hole is punched out of the cap and a rubber washer is inserted in place of the waxed millboard. In order to protect the surface of the cap and the exposed portion of the rubber washer from contamination before use, the cap and neck of the bottle are covered by a viskap,¹ such as is seen on perfume bottles. This is a cellulose preparation which is slipped on moist and allowed to dry. In

¹ Made by the Viscose Development Co. Ltd., Woldham Road, Bromley, Kent.

so doing the viskap shrinks, moulding itself tightly to the cap and neck of the bottle.

The apparatus is fitted up as follows :—

The bottles arrive in a carton already washed, cleaned and capped, so that no further preparation is required. The caps are white, having been sprayed with a cellulose paint, and it is better to remove this with a little acetone, or by placing the caps in a weak solution of lysol. The waxed millboard washer is removed and a $\frac{1}{4}$ in. hole punched out of the centre of the cap by means of a hollow punch. A rubber washer $1\frac{1}{8}$ in. in diameter is cut, either by means of a hollow punch or by clipping the rubber with scissors round a farthing, and fitted into the cap. The sheet rubber used is motor-car inner tube, and stands autoclaving without change. In order to prevent the rubber washer from adhering to the rim of the bottle, a "blackol" liner is placed over it.

The medium in the bottle is a matter of choice, and the following range is useful. The different types are recognised by the colour of the viskap and the glass bead in the bottle. (1) Plain broth, white cap and bead ; (2) broth + 0·1 per cent. glucose, green cap and bead ; (3) broth + 0·2 per cent. sodium citrate, purple cap and bead ; (4) broth + 0·5 per cent. bile salt, yellow cap and bead. The size of the viskaps is No. 2 semi-opaque cut $1\frac{3}{8}$ in. These are dyed by placing them in a small quantity of the required colour—*e.g.* malachite green, methyl violet, acriflavine—after which they are washed in warm water. The bead is to identify the medium on its return to the laboratory if the viskap has been entirely removed.

100 c.c. of the medium are placed in the bottle, the appropriately coloured bead added, and the perforated cap with rubber washer and "blackol" liner firmly screwed on. The bottle is now sterilised in the autoclave for fifteen minutes at 5 lbs. pressure, and when cool the appropriately coloured viskap is at once slipped on. The viskaps dry in a few hours and mould themselves to the cap and neck of the bottle. The broth can be stored without deterioration. For streptococci or pneumococcus the glucose broth will be found the most useful. The bile-salt broth is intended for cases of suspected enteric fever, but its use is not essential.

For use, the bottle is taken to the bedside of the patient. Just before the vein is punctured the edge of a penknife is run round the screw cap to sever the viskap just above the flange. The point of the knife is then gently inserted under the upper half of the viskap, which can be flicked off without touching the top of the screw cap. Blood (5–10 c.c.) is now withdrawn from the vein, with the usual precautions, and immediately afterwards the needle is passed through the rubber washer,

and the blood is expelled into the medium. The needle is withdrawn and the puncture in the washer seals itself. The bottle is shaken to mix blood and broth, and sent to the laboratory for incubation. It is advisable to wipe the exposed portion of the washer with a little antiseptic (*e.g.* spirit), in order to remove any infective material at the site of the puncture.

When the culture is to be examined, after incubation, the screw cap is removed in the ordinary way.

After incubation for eighteen to twenty-four hours, films are made from the broth and stained by Gram's method. If organisms are noted, sub-inoculations are made on nutrient-agar slopes or an agar plate by the successive stroke method (*vide* p. 132). The sub-cultures are incubated and the organisms developing



Venule before use.



Sectional view—ready for use.



Sectional view - valve opened in use.

(Reproduced by permission of Bayer Products Ltd.)

are identified as far as possible by their microscopic characters and colony appearances. If further investigations are required for accurate identification, single colonies are "picked off" on to agar slopes and the resulting cultures are studied.

Where an infection with the enteric group is suspected, it is convenient to plate directly from the blood culture on MacConkey's medium to elicit the characteristic colonies on this medium.

Even when no organisms can be detected in films from the primary blood culture, it is advisable to

make sub-inoculations, as scanty organisms may not be observed, but still develop colonies in subculture.

If no result is obtained after twenty-four hours' incubation, the blood-broth should be incubated continuously for *at least* four days, films and sub-inoculations being made each day.

A most convenient instrument for drawing aseptically a blood sample from a vein is the so-called *Behring Venule*.¹ It also serves as a container for transmitting the sample to the laboratory, and provides medical practitioners with a simple means of carrying out the clinical technique of blood culture. It consists of an evacuated receiving-tube closed by a hollow rubber stopper, and a needle attached to a narrow glass connecting-tube which passes through the stopper and is kept closed by a simple valve arrangement (see diagrams, p. 144). The needle is enclosed in a sealed extension of the connecting-tube. Both the needle and interior of the receiving-tube are sterile. The needle is exposed by breaking off the covering tube and is inserted into a vein; the valve is then opened by bending the "knee" formed by the receiving and the connecting tubes. Blood is automatically drawn into the receiving-tube and thereafter the valve is allowed to close. In addition to the "venule" for collecting ordinary blood samples, special "venules" are obtainable—*e.g.* containing broth for blood culture, bile for blood culture in enteric fever, glass beads to defibrinate the blood, citrate solution to obviate coagulation, etc.

DISPOSAL OF CULTURES

Cultures to be discarded should be killed by heat or antiseptics before the container is cleaned for re-use.

In the case of non-sporing organisms, it is sufficient to remove the cotton-wool plugs, and immerse the tubes and plugs in a large basin of 3 per cent. lysol or cresol.

When 1 oz. screw-capped bottles are used as test-tubes, the cap is completely unscrewed, and it and the bottle placed in the lysol solution.

Cultures of sporing organisms, such as B. tetani, B. anthracis, etc., should be sterilised by autoclaving.

¹ These instruments can be obtained from Bayer Products Ltd., London.

PERSONAL PRECAUTIONS IN BACTERIOLOGICAL LABORATORY WORK

It is essential to wear an overall while at work. If any material containing pathogenic organisms drops on the bench, floor, clothes, apparatus, etc., it should be sterilised at once with 1:1000 perchloride of mercury solution or 3 per cent. lysol. If the hands become contaminated they should be sterilised in a basin of dilute lysol or perchloride of mercury solution, and workers should make it a rule always to sterilise and wash the hands after completing any bacteriological work.

Precautions to be taken in carrying out special methods are referred to later.

CHAPTER V

STAINING METHODS

As bacteria consist of clear protoplasmic matter, differing but slightly in refractive index from the medium in which they are grown, it is difficult, except by special methods of illumination, to see them in the unstained condition.

Staining, therefore, is of importance, not only for the recognition of bacteria, but also in virtue of the fact that by special methods certain organisms, such as tubercle and diphtheria bacilli, may be differentiated microscopically from others.

Bacterial protoplasm reacts to stains in a manner similar to the nuclear material of tissue cells, and therefore the various basic aniline dyes are the commonest stains employed. The action of these stains may be intensified by the use of mordants such as phenol or weak alkalies, by the application of heat, or by prolonging the time of staining. Some organisms have a greater affinity for dyes than others, so that when stained and then treated with a decolorising agent they still retain the stain while others lose it. By such means it is often possible to differentiate varieties of bacteria in a mixture.

METHODS OF MAKING FILM OR SMEAR PREPARATIONS

Before taking up the various staining processes, details of the methods employed in making films must be considered.

Film preparations are made either on cover-slips or on the ordinary 3×1 in. glass slides. It is essential that

the cover-slips or slides should be perfectly clean and free from grease, otherwise uneven films will result.

Cover-Slips.—These should be $\frac{3}{4}$ or $\frac{1}{2}$ in. square, and of No. 1 thickness. (Thicker cover-slips—No. 2—prevent the oil-immersion objective from coming near enough for the specimen to be focussed.) They may be cleaned by placing them in a mixture of sulphuric acid, 6 parts; potassium bichromate, 6 parts; water, 100 parts. They should be dropped one by one into the fluid. The solution is contained in an evaporating dish and boiled. The cover-slips are then well washed, first in tap water and then in distilled water, and stored in a stoppered jar in 50 per cent. spirit. Before use they are dried with a soft clean cloth, such as an old handkerchief.

Slides.—These may be treated in a manner similar to cover-slips. A quicker and quite satisfactory method for ordinary routine use is to moisten the finger with water, rub it on the surface of some fine sand soap such as “Monkey Brand” or “Bon Ami,” and then smear the surface of the slide. After removing the soapy film with a clean cloth the surface is clean and free from grease. If the slide is perfectly clean a drop of water can be spread over its surface in a thin even film, whereas if it is greasy the water collects into small drops and a film cannot be made.

After the films have been made and examined the slides should be discarded. They should not be cleaned and used again.

In the case of fluid materials, such as broth cultures, urine, sputum, pus, etc., one loopful (or more) is taken up with the inoculating wire and is spread thinly over the slide. A little experience will soon determine the amount required, and in spreading the films it will be found that there are both thick and thin portions. The slide is then held in the palm of the hand over a Bunsen flame and dried. The film is fixed either by passing the slide three times slowly through the flame, or by heating through the glass slide. In the latter method the slide is held, film upwards, in

the top of the Bunsen flame for a few seconds so that the slide becomes hot. Care must be taken not to char the film, and when the slide is just too hot to be borne on the back of the hand, fixation is complete.

Films on cover-slips require a minimum of time for fixing owing to the thinness of the glass. The cover-slips may be held by means of Cornet's forceps.

With solid material, such as cultures on agar, etc., it is necessary to place a loopful of clean water on the slide. The loop is then sterilised and a minute quantity of material, obtained by just touching the growth, is transferred to the drop, thoroughly emulsified, and the mixture is spread evenly on the slide. The resulting film is fixed and dried as above. *Beginners are very apt to take more material than necessary from the culture and thus make too thick films.*

STAINING

The method of staining varies with the nature of the preparation (film or section).

FILMS

The stains are poured directly or filtered on to the slide. When staining is completed, the dye is washed off with water and the slide is allowed to dry in the vertical position or is placed between two sheets of white fluffless blotting-paper or filter paper. The drying of the film is completed over the Bunsen flame. Such stained films may be mounted in Canada balsam under a cover-slip, or may be examined unmounted with the oil-immersion lens, a small drop of cedar-wood oil being placed directly on the film. If it is desired to mount the preparation later, the oil can be removed with xylol.

TISSUE SECTIONS

The sections being embedded in paraffin (*vide* p. 177), it is necessary to remove the paraffin so that a watery stain may penetrate. The paraffin is first removed

with xylol,¹ the xylol removed with alcohol, and the alcohol replaced by water. The staining process is then proceeded with. After staining, the section must be dehydrated with absolute alcohol, then cleared in xylol and finally mounted in Canada balsam under a cover-slip. The Canada balsam (which is a resin) is dissolved in xylol in order to render it of suitable consistence.

Technique.—The slide bearing the paraffin section is placed in a jar of xylol for some minutes to remove the paraffin. The section is then treated with a few drops of 95 per cent. alcohol, when it immediately becomes opaque. A few drops of 50 per cent. spirit are poured on, and the slide is finally washed gently in water. If the tissue has been fixed in any corrosive sublimate preparation, such as Zenker's fluid, the section should be treated with Gram's iodine solution for a few minutes (*vide* p. 153), then with spirit and finally water. The sections are now ready to be stained. After staining and washing with water, the slide is wiped all round the section with a clean duster to remove excess of water. The bulk of the water in the section may be removed by pressing between fluffless blotting-paper. The section is *immediately* treated with a few drops of spirit, then absolute alcohol. The slide is again wiped all round the section, a few more drops of absolute alcohol are poured on, and the slide is then immersed in xylol. When cleared, the slide is removed, and excess of xylol round the section is wiped away, a drop of Canada balsam is applied and the section mounted under a No. 1 cover-slip. It is essential that the section should not be allowed to dry at any period of the process, and that dehydration with absolute alcohol should be complete in order that the section may be thoroughly cleared.

When the bacteria are readily decolorised by alcohol, aniline-oil-xylol (aniline oil, 2 parts; xylol, 1 part) should be used for dehydration. After wash-

¹ Benzol may be substituted for xylol.

ing, when the slide has been wiped round the section, the preparation is blotted and then treated with the aniline-oil-xylol mixture, which clears as well as dehydrates. The aniline-oil-xylol is then replaced by xylol. This can be done conveniently by holding the slide almost vertically and dropping xylol from a drop bottle on to the slide just above the section. The xylol flows over the section and quickly removes the aniline oil. The preparation is mounted immediately in Canada balsam.

SIMPLE STAINS

These show not only the presence of organisms, but also the nature of the cellular content in exudates.

METHYLENE BLUE

Of the many preparations of this dye, Löffler's methylene blue is perhaps the most useful:

Saturated solution of methylene blue in alcohol	30 c.c.
Solution of caustic potash in water (1 : 10,000)	100 c.c.

(This caustic potash solution is made by adding 1 c.c. of a 1 per cent. solution to 99 c.c. of water)

Films.—Stain for three minutes, then wash with water. This preparation does not readily over-stain.

Sections.—Stain for five minutes or longer. The application of the alcohol during dehydration is sufficient for differentiation. Aniline-oil-xylol can also be used for dehydration and clearing.

POLYCHROME METHYLENE BLUE

This is made by allowing Löffler's methylene blue to "ripen" slowly. The stain is kept in bottles, which are half filled and shaken at intervals to aerate thoroughly the contents. The slow oxidation of the methylene blue forms a violet compound which gives the stain its polychrome properties. The ripening takes twelve months or more to complete. The preparation is used in a manner similar to Löffler's

methylene blue; it is employed in McFadyean's reaction (*vide* p. 314).

CARBOL THIONIN

Stock Solution :

Thionin	1 gram
Carbolic acid 1 : 40 watery solution	100 c.c.

For Use :

Stock solution	1 part
Distilled water	3 parts

(Filter before use)

This method is useful for demonstrating in tissues such organisms as typhoid and glanders bacilli.

- (1) Stain sections five to ten minutes.
- (2) Wash well with water.
- (3) Differentiate in a bowl of water to which a few drops of acetic acid have been added.
- (4) Wash well with water.
- (5) Blot, dehydrate with absolute alcohol, clear in xylol and mount in Canada balsam.

DILUTE CARBOL FUCHSIN

Made by diluting Ziehl-Neelsen's stain (p. 158) with ten to fifteen times its volume of water. Stain for ten to twenty-five seconds and wash well with water. Over-staining must be avoided, as this is an intense stain, and prolonged application colours the cell protoplasm in addition to nuclei and bacteria.

GRAM'S STAINING METHOD

This is one of the most important methods in bacteriology, and must be employed for the diagnostic identification of certain types, such as the gonococcus.

The principle of the method is as follows:—certain bacteria when treated with one of the para-rosaniline dyes such as methyl violet or gentian violet, and then with iodine, “fix” the stain so that subsequent treatment with a decolorising agent—*e.g.* alcohol or aniline-oil—does not remove the colour. Other organisms, however, are decolorised by this process

(*vide* p. 157). If a mixture of various organisms be thus stained and subjected to the decolorising agent, it will be found that some varieties retain the dye, and these are termed "Gram-positive," whereas others are completely decolorised and are designated "Gram-negative." In order to render the decolorised organisms evident, and to distinguish them from those retaining the colour, a contrast or counter-stain is then applied. This contrast stain is usually red, in order that the Gram-negative organisms may easily be differentiated from the Gram-positive organisms, which retain the original violet stain. Gram's method can be carried out only with the pararosaniline dyes—*e.g.* methyl violet, crystal violet, gentian violet (which is a mixture of the two preceding dyes) and victoria blue. Methyl violet and gentian violet are the usual stains employed, but crystal violet is a purer stain and can be used to advantage instead of them.

WEIGERT'S MODIFICATION

Solutions employed—

(1) Carbol Gentian Violet¹:

Saturated alcoholic solution of gentian violet	1 part
5 per cent. solution of carbolic acid in distilled water	10 parts

(This mixture should be made up each day, as it tends to precipitate)

(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 :

Ziehl-Neelsen's carbol fuchsin (p. 158)	1 part
Distilled water	9 parts

¹ While gentian violet is the original stain for Weigert's modification it is strongly recommended that crystal violet be used instead.

PROCEDURE

(a) *Films*.—The film is made, dried, and fixed in the usual manner.

- (1) Stain film with carbol gentian violet (two to three minutes).
- (2) Pour off stain, replace with Gram's iodine solution, and allow to act for one minute.
- (3) Dry thoroughly by blotting and gentle heat.
- (4) Decolorise with aniline-oil-xylol, using several changes until the stain ceases to be removed.

Breathing on the slide after the first application of aniline oil hastens decolorisation.

Now examine at this stage under the low power of the microscope; the nuclei of the pus cells should be of a pale-violet colour; if the nuclei are deeply stained, then decolorisation is incomplete.

- (5) Wash with several changes of xylol and dry.
- (6) Counter-stain with dilute carbol fuchsin ten to twenty-five seconds. Wash with water and dry.

(b) *Sections*.—Counter-stain first with dilute carbol fuchsin, 1:10, for fifteen seconds, and then proceed as above. After (5), the sections will be cleared, and can at once be mounted in Canada balsam.

JENSEN'S MODIFICATION

This modification can be recommended particularly to those commencing staining methods.

Solutions required—

- (a) Methyl violet, 6 B.,¹ 0·5 per cent. solution in distilled water.

(The solution should be made up in bulk and filtered. It keeps indefinitely, and does not precipitate, but should be filtered again before use)

¹ Crystal violet in the same proportion is recommended as an alternative.

(b) Iodine Solution :

Iodine	1 gram
Potassium iodide.	2 grams
Distilled water	100 c.c.

Note that the iodine solution is three times stronger than the original Gram's iodine.

(c) Counter-stain.—Neutral Red Solution :

Neutral red	1 gram
1 per cent. acetic acid	2 c.c.
Distilled water	1000 c.c.

Film Preparations.—Smears are made, dried, and fixed in the usual way.

- (1) Pour on methyl violet solution and allow to act for twenty to thirty seconds.
- (2) Pour off excess of stain, and, holding the slide at an angle downwards, pour on the iodine solution so that it washes away the methyl violet. Allow the iodine to act for a half to one minute.

Note.—It is important that the iodine should wash away the methyl violet, as any mixture of the two solutions causes a precipitate which deposits on the slide.

- (3) Wash off the iodine with spirit, and treat with fresh spirit until colour ceases to come out of the preparation. This is easily seen by holding the slide against a white background.
 - (4) Wash with water.
 - (5) Apply counter-stain for thirty to sixty seconds.
 - (6) Wash with water and dry between blotting-paper.
- This method is very simple, and gives excellent results with freedom from deposit.

Dilute carbol fuchsin (1:15) applied for twenty to thirty seconds may be substituted with advantage as a counter-stain for routine work, but for demonstrating the gonococcus and other intracellular Gram-negative bacteria the neutral-red counter-stain should be used.

KOPELOFF AND BEERMAN'S MODIFICATION¹

The following method has been found useful for class work and is recommended for beginners :

¹ N. Kopeloff and P. Beerman, *Proc. Soc. for Exp. Biology*, 1922-1923, vol. 20, p. 71.

Films—

- (1) Make a thin film, dry in the air and fix with the least amount of heat necessary to kill the organisms.
- (2) Flood slide with stain made up as follows :

Stain :

1 per cent. aqueous solution of methyl violet, 6 B.	30 parts
5 per cent. solution of sodium bicarbonate	8 parts

Allow to remain on the slide for five minutes or more.

(The above solution is apt to precipitate within a few days; it acts quite well without the addition of the bicarbonate solution and will keep indefinitely)

- (3) Wash off excess of stain with iodine solution and allow to act for two minutes.

Iodine Solution :

Iodine	2 grams
Normal solution of sodium hydroxide	10 c.c.
Distilled water.	90 c.c.

- (4) Drain off the excess of iodine and add acetone (100 per cent.) drop by drop until no colour is seen in the washings.

(It should be noted that decolorisation is very rapid, requiring ten seconds or less, and the time should be reduced to a minimum)

- (5) Wash slide in water.
- (6) Counter-stain for ten to thirty seconds with 0.05 per cent. aqueous solution of basic fuchsin.
- (7) Wash in water, blot, and dry in the air.

Sections—

- (1) Remove paraffin in benzol or xylol.
- (2) Treat the section with spirit and wash in water.
- (3) Flood with the stain and allow to act for five minutes.
- (4) Wash off excess of stain with the iodine solution and allow to act for two minutes.
- (5) Decolorise with acetone (*vide supra*).
- (6) Wash slide in water.
- (7) Counter-stain for ten to thirty seconds.
- (8) Wipe carefully around the section to remove as much water as possible, dehydrate quickly in absolute alcohol, clear in benzol or xylol and mount in Canada balsam.

If Gram's method is properly carried out, Gram-positive organisms and fibrin are stained dark violet in colour. Gram-negative organisms, the nuclei and protoplasm of pus cells and tissues cells are stained pink with the counter-stain.

To obviate errors from over-decolorising, a film of a known Gram-positive organism (*e.g.* a pure culture of *Staphylococcus aureus*) may be made at one side of the pus film. This "control spot" is stained along with the film. For the recognition of Gram-negative organisms, such as gonococci or meningococci in pus, the "control-spot" must retain the violet stain while the nuclei of the pus cells are stained only with the counter-stain.

REACTIONS OF CERTAIN ORGANISMS TO GRAM'S STAIN

<i>Positive</i>	<i>Negative</i>
Staphylococcus	Gonococcus
Streptococcus	Meningococcus
Pneumococcus	Diplococcus catarrhalis
Micrococcus tetragenus	B. melitensis and B. abortus
B. diphtheriae and group of diphtheroid bacilli	Pneumobacillus of Friedländer
Tubercle bacillus. (This requires a special method, as the organism is <i>not usually stained</i> by the ordinary Gram's method)	Koch-Weeks bacillus
Smegma bacillus	Diplo-bacillus of Morax
Leprosy bacillus	B. proteus
Streptothrix actinomyces	B. pyocyaneus
B. anthracis	B. mallei
B. tetani	The coli-typhoid group, B. enteritidis Gaertner and allied organisms, and the dysentery group of bacilli
B. welchii	Vibrios— <i>e.g.</i> V. cholerae
B. sporogenes	B. pestis
Vibrio septique	B. influenzae (Pfeiffer)
B. botulinus	B. pertussis
	B. fusiformis
	Spirochaetes
	Bacterium pneumosintes

Note.—The above table refers only to young cultures, as degenerated organisms belonging to the Gram-positive class may react negatively.

STAINING OF TUBERCLE AND OTHER ACID-FAST BACILLI

ZIEHL-NEELSEN METHOD

The tubercle bacillus does not stain with the ordinary aniline dyes, on account of a resistant outer envelope of a fatty nature which prevents penetration of the stain. The principle of staining this organism is the following:—

By the use of a powerful staining solution which contains a mordant, and the application of heat, the dye can be made to penetrate the bacillus. Just as the organism is resistant to ordinary stains, it also tends to resist decolorisation. Once stained, the tubercle bacillus will withstand the action of powerful decolorising agents for a considerable time and thus still retains the stain when everything else has been decolorised.

The stain used consists of basic fuchsin, with phenol as the mordant. The dye is basic and its combination with an acid produces a compound which is yellowish-brown in colour. Any strong acid can be used as a decolorising agent, but sulphuric acid in a 20 per cent. solution is usually employed.

In order to show what has been decolorised, and to form a contrast with the red-stained bacilli, the preparation is counter-stained with methylene blue.¹

Ziehl-Neelsen's (strong) Carbol Fuchsin :

Basic fuchsin	1 part
Absolute alcohol	10 parts
Solution of carbolic acid (1 : 20).	100 parts

Dissolve the dye in the alcohol and add the solution to the carbolic acid.

¹ Malachite green is recommended as a counter-stain in the Ziehl-Neelsen method. A stock solution of 1 per cent. in distilled water is made, and for use a small quantity is diluted with distilled water in a drop bottle so that fifteen to twenty seconds' application of the weak stain gives the background a pale green tint. Deep counter-staining must be avoided. The pale green background is pleasant for the eyes, and is thought by some workers to make scanty organisms more easily noticed.

Films.—Smears are made, dried and fixed in the usual manner :

- (1) Flood the slide with filtered carbol fuchsin and heat until steam rises. Allow the preparation to stain for five minutes, heat being applied at intervals to keep the stain hot. The stain must not be allowed to evaporate and dry on the slide.
- (2) Wash with water.
- (3) Immerse the slide in 20 per cent. sulphuric acid. The red colour of the preparation is changed to yellowish brown. After about a minute in the acid remove the slide, wash with water and place it in the acid again. This process should be repeated several times. The object of the washing is to remove the compound of acid with stain and allow fresh acid to gain access to the preparation. The decolorisation is finished when, after washing, the smear is a faint pink.
- (4) Wash the slide well in water.
- (5) Treat with 95 per cent. alcohol for two minutes.
- (6) Wash with water.
- (7) Counter-stain with Löffler's methylene blue or dilute malachite green for ten to thirty seconds.
- (8) Wash, blot, dry and mount.

Acid-fast bacilli stain bright red, while the tissue cells and other organisms are stained blue.

Other organisms are " acid-fast " in addition to the tubercle bacillus. The most important in diagnostic work is the smegma bacillus, which is frequently found in samples of urine. Treatment with spirit in addition to acid may, however, decolorise this type of organism, whereas the tubercle bacillus is both acid- and alcohol-fast. The decolorisation with spirit is therefore important when examining urine for the presence of the tubercle bacillus.

It should be noted that in films stained by Ziehl-Neelsen's method, red-stained organisms in the midst of hyaline material must not be regarded as tubercle bacilli, as such material may be resistant to decolorisation.

Sections.—

- (1) Sections are treated with xylol to remove paraffin, then with spirit, 50 per cent. alcohol, and finally washed in water.
- (2) Stain with Ziehl-Neelsen's stain as described for films, but heat gently, otherwise the section may become detached from the slide.
- (3) Wash with water.
- (4) Decolorise with 20 per cent. sulphuric acid as for films. The process takes longer owing to the thickness of the section, and care must be exercised in washing, to retain the section on the slide.
- (5) Wash well with water.
- (6) Counter-stain with methylene blue or malachite green for a half to one minute.
- (7) Wash with water.
- (8) Wipe the slide dry all round the section, blot with filter paper or fluffless blotting-paper, and treat with a few drops of absolute alcohol. Pour on more absolute alcohol, wipe the slide again and immerse it in the xylol jar.
- (9) Mount in Canada balsam.

Leprosy bacilli are also acid-fast, but not to the same extent. They are stained in smears or sections in the same way as the tubercle bacillus, except that 5 per cent. sulphuric acid is used for decolorisation.

STAINING OF DIPHTHERIA BACILLUS

The diphtheria bacillus gives its characteristic staining reactions only in a young culture (eighteen to twenty-four hours) on a serum medium (*vide* p. 282).

NEISSER'S METHOD (Modified)

The following modification of Neisser's method gives better results than the original, and is recommended for general use :—

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

Counter-Stain :

(a)	Chrysoidin	.	.	.	1 gram
	Distilled water	.	.	.	300 c.c.
	(Dissolve by gentle heat, and filter)				
or (b)	Bismarck-brown	.	.	.	1 gram
	Distilled water	.	.	.	500 c.c.

- (1) Mix together two parts of solution A and one part of solution B. Stain films in the mixture for a few seconds.
- (2) Wash in water.
- (3) Counter-stain with chrysoidin or Bismarck-brown for fifteen seconds.
- (4) Wash in water, blot and dry.

The volutin granules of the diphtheria bacillus appear bluish-black, while the protoplasm is stained yellowish-brown.

PUGH'S STAIN

Toluidin blue	.	.	.	.	2 grams
dissolved in absolute alcohol	.	.	.	.	20 c.c.
and added to glacial acetic acid	.	.	.	.	50 c.c.
in distilled water	.	.	.	.	950 c.c.

The dried film is flooded with stain and heated till steam rises. Stain for one to three minutes. The preparation will not over-stain. Wash with water and dry.

The protoplasm of the bacilli is light blue and the granules are coloured reddish-purple.

ALBERT'S STAIN¹

Laybourn's modification,² in which malachite green is substituted for methyl green, is given here instead of the original method.

¹ *Journ. Amer. Med. Assoc.*, 1921, vol. 76, p. 240.

² *Ibid.*, 1924, vol. 83, p. 121.

Solution 1

Toluidin blue	.	.	.	.	.	0.15 gram
Malachite green	.	.	.	.	.	0.20 gram
Glacial acetic acid	.	.	.	.	.	1.0 c.c.
Alcohol (95 per cent.)	.	.	.	.	.	2.0 c.c.
Distilled water	.	.	.	.	.	100 c.c.

Dissolve the dyes in the alcohol and add to the water and acetic acid.

Allow to stand for one day and then filter.

Solution 2

Iodine	.	.	.	.	.	2 grams
Potassium iodide	.	.	.	.	.	3 grams
Distilled water	.	.	.	.	.	300 c.c.

Note.—The iodine solution used in Jensen's modification of Gram's method (p. 155) works equally well.

Technique—

Make smear preparation and fix by heat.

Apply solution 1 for three to five minutes.

Wash in water and blot dry.

Apply solution 2 for one minute.

Wash and blot dry.

By this method the granules stain bluish-black, the protoplasm green and other organisms mostly light green.

We strongly recommend this method for general routine use.

STAINING OF SPORES

If spore-bearing organisms are stained by ordinary dyes, the body of the bacillus is deeply coloured, whereas the spore is unstained and appears as a clear area in the organism. This is because the spore has an outer envelope which prevents the stain from penetrating the protoplasm in a manner similar to, but not to the same extent as, the tubercle bacillus (*q.v.*). Once the spores are stained, they tend to retain the dye after treatment with decolorising agents.

The following is a simple and satisfactory method for staining spores:—

Films, which must be thin, are made, dried and

fixed in the usual manner with the minimum amount of heating.

- (1) Stain with Ziehl-Neelsen's carbol fuchsin for three to five minutes, heating the preparation until steam rises.
- (2) Wash in water.
- (3) Treat with 5 per cent. sodium sulphite solution for a few seconds.
- (4) Wash with water.
- (5) Counter-stain with Löffler's methylene blue for eight minutes.
- (6) Wash in water, blot and dry.

The spores are stained bright red and the protoplasm of the bacilli blue.

It should be noted that the spores of some bacteria are decolorised more readily than those of others.

STAINING OF CAPSULES

Two methods are given. The first, Hiss's method, is useful for general routine work, and the second, Richard Muir's method, gives beautiful results, but is much more difficult for the beginner and is not recommended for general use.

HISS'S METHOD

Solutions required—

- | | |
|---|----------|
| (1) Saturated alcoholic solution of gentian violet ¹ | 1 part |
| Distilled water | 19 parts |
| (2) Copper sulphate solution 20 per cent. in distilled water. | |

Films should be thin and fixed by heat in the usual manner.

- (1) Pour on the stain and gently heat until steam rises. Allow the stain to act for fifteen to twenty seconds.
- (2) Wash off the stain with the 20 per cent. copper sulphate solution, without washing in water,

¹ Basic fuchsin may be substituted for gentian violet.

dry the film between blotting-paper, and mount, if necessary, in balsam.

In order to avoid an excessive deposit of copper sulphate crystals on the film, successive amounts of copper sulphate solution should be poured on the film until the slide is quite cool.

The bacteria are stained deep violet, while the capsules are pale violet in colour.

R. MUIR'S METHOD

Solutions required—

Strong carbol fuchsin.

Special mordant.

Löffler's methylene blue.

Mordant :

Saturated solution of corrosive sublimate . . . 2 parts

20 per cent. solution of tannic acid . . . 2 parts

Saturated solution of potassium alum . . . 5 parts

The film, which must be very thin, is dried and fixed by heat.

(1) Stain with strong carbol fuchsin for one minute, the preparation being gently heated.

(2) Wash slightly with spirit and then well in water.

(3) Pour on the mordant and allow it to act for thirty seconds.

(4) Wash well with water.

(5) Treat with spirit. The time varies with the preparation and is found by trial. About thirty to forty seconds is ample, and the film should be pale red in appearance.

(6) Wash well in water.

(7) Counter-stain with Löffler's methylene blue for a half to one minute.

(8) The preparation may be washed and dried in the usual manner,

or dehydrated with absolute alcohol, cleared in xylol and mounted in balsam. This gives somewhat clearer specimens.

The bacteria are bright red, and the capsules are bright blue.

STAINING OF FLAGELLA

Thin films are made from agar cultures. A small amount of the culture is emulsified in water, the quantity of culture being only as much as will cause

the faintest turbidity of the water. A film is made from a drop of the emulsion on a clean slide and allowed to dry in the air. Strict attention should be paid to the cleanliness of the slides, which are treated with the bichromate-sulphuric acid solution described on p. 148, well washed, and carefully dried. They are then passed through the Bunsen flame and allowed to cool before spreading the films.

J. KIRKPATRICK'S METHOD ¹

Solutions required—

(1) *Fixing solution :*

Absolute alcohol		60 c.c.
Chloroform		30 c.c.
Formalin		10 c.c.

(2) *Mordant :*

Ferric chloride, 5 per cent. solution		1 part
Tannic acid, 20 per cent. solution (dissolved by heat and allowed to cool)		3 parts

Before use, dilute the mordant with an equal quantity of water.

(3) *Silver solutions :*

(a) *Silver stock solution.*—Add 200 c.c. distilled water to 10 grams of silver sulphate ² in a screw-capped medical flat bottle, and incubate for twenty-four hours at 37° C., shaking occasionally. This solution keeps well.

(b) *Silver staining solution.*—Rinse a clean 100 c.c. conical flask with distilled water. Place 40 c.c. of the filtered silver stock solution in the flask, and add quickly 0.6 c.c. ethylamine “33 per cent. W.V.” ³. A precipitate forms which is immediately dissolved. From a clean drawn-out pipette add filtered silver stock solution until the solution remains permanently opalescent. Now add 10 c.c. distilled water.

¹ Method described in ninth edition of Muir and Ritchie's *Manual of Bacteriology*.

² British Drug Houses Ltd.

³ W.V. = Weight-Volume—*i.e.* 33 per cent. by weight of ethylamine.

Method

Use agar cultures thirty-six to forty-eight hours old. Heat a clean $3 \times \frac{1}{2}$ -in. test-tube and allow to cool. Suspend a loopful of the agar culture in 1 c.c. sterile distilled water in the tube, rotating the loop very gently. Add distilled water until the suspension is faintly opalescent.

- (a) Place a loopful of the suspension on a clean slide and draw the excess to one side by means of the platinum wire, so that a thicker part of the film (which acts as a control spot in the staining process) is formed. Allow the film to dry in the air.
- (b) Treat the film with the fixing solution for one to three minutes.
- (c) Rinse in spirit and wash thoroughly in water.
- (d) Treat with the mordant for three to five minutes.
- (e) Wash well with water and dry the under surface of the slide.
- (f) Filter on the silver staining solution and heat gently until the thick control spot becomes dark brown in colour, and a metallic scum appears on the edges of the fluid—about fifteen seconds. Cease heating and allow the solution to act for a further fifteen to thirty seconds.
- (g) Wash off the staining solution in running water (do not pour off).
- (h) Dry the film, and mount in balsam.

If the staining is successful the organisms are stained black, while the flagella are clearly defined and are light brown or grey in colour. A granular appearance in the flagella may be due to excess of ethylamine, over-heating, or over-treatment in the mordant. Too little ethylamine or excessive heating may cause a crystalline deposit. If the directions are carefully followed the method gives good results.

THE ROMANOWSKY STAINS

The original Romanowsky stain was made by dissolving in methyl alcohol the compound formed by the interaction of watery solutions of watery cosin and zinc-free methylene blue. The original stain has now been replaced by various modifications which are easier to use and give better results; these are:—Leishman's, Wright's, Jenner's and Giemsa's stains. The peculiar property of the Romanowsky stains is that they impart a reddish-purple colour to the chromatin of malaria and other parasites. This colour is due to a substance which forms when methylene blue is "ripened," either by age, as in polychrome methylene blue, or by heating with sodium carbonate. The latter method is employed in the manufacture of Leishman's and Wright's stains. The ripened methylene blue is mixed with a solution of water-soluble eosin, when a precipitate, due to the combination of these dyes, is formed. The precipitate is washed with distilled water, dried, and dissolved in methyl alcohol¹ *which must be acetone-free*. Each modification of the Romanowsky stain varies according to the "ripening" and the relative proportions of methylene blue and eosin.

According to the nature of the microscopic preparation, different stains are employed. Thus for the cytological examination of blood, Jenner's stain is used; for the malaria parasite and trypanosomes, Leishman's and Wright's modifications give the best results, while the pathogenic spirochaetes (particularly the *Treponema pallidum* of syphilis) and certain protozoa can be demonstrated best by Giemsa's stain.

The Romanowsky stains, except Jenner's stain, are usually diluted for staining purposes with distilled water, when a precipitate is formed which is removed by subsequent washing.

¹ The methyl alcohol must be "pure, for analysis," and have a pH of 6.5. If too acid, the reaction must be adjusted (with the usual indicators) by the addition of N/100 NaOH.

JENNER'S STAIN

This can be purchased ready for use, but may be made by dissolving 0·5 gram of powdered stain in 100 c.c. acetone-free methyl alcohol. This stain is satisfactory for the cytological examination of blood films, but is not so suitable for parasites.

The stain is poured on the dried but *unfixed* film and allowed to act for one to three minutes. The film is then washed with distilled water until pink. The slide is blotted and allowed to dry in the air.

LEISHMAN'S STAIN

This stain may be purchased ready for use or made by dissolving 0·15 gram of Leishman's powder in 100 c.c. acetone-free methyl alcohol. The powder is ground in a mortar with a little methyl alcohol, the residue of undissolved stain allowed to settle and the fluid decanted into a bottle. The residue in the mortar is treated with more methyl alcohol, and the process is repeated until all the stain goes into solution. The remainder of the methyl alcohol is now added. The stain is improved by keeping two weeks before use.

Films.—Dry unfixed films are used. The stain is first used undiluted, and the methyl alcohol fixes the film. The stain is then diluted with distilled water, and the staining proper carried out.

- (1) Pour the undiluted stain on the unfixed film and allow it to act for one minute and a half.
- (2) By means of a pipette and rubber teat add double the volume of distilled water to the slide, mixing the fluids by alternately sucking them up in the pipette and expelling them. Allow the diluted stain to act for five to seven minutes.
- (3) Wash the slide gently with distilled water, allowing the preparation to differentiate in the distilled water until the film appears bright pink in colour—usually about a minute.
- (4) Remove the excess of water with blotting-paper and dry in the air.

It is important that the reaction of the distilled water be neither acid nor alkaline. Any slight variations from neutrality

may alter considerably the colour of granules in white blood corpuscles, etc., and give rise to supposed "pathological" appearances in cells which are really normal. A simple method of ensuring a suitable reaction of the distilled water is to keep large bottles of it—e.g. aspirator bottle—specially for these stains. Add 2 or 3 drops of 1 per cent. aqueous neutral-red solution. The usual reaction of distilled water is slightly acid, and a few drops of 1 per cent. sodium carbonate solution should be added until the solution shows the faintest possible suggestion of pink colour.

When the staining is excessively bluish, as in old films, good differentiation is obtained by brief washing with 1 per cent. mono-sodium phosphate.

Sections—

- (1) The section is treated with xylol to remove the paraffin, then with alcohol, and finally distilled water.
- (2) Drain off the excess of water and stain for five to ten minutes with a mixture of 1 part stain and 2 parts of distilled water.
- (3) Wash with distilled water.
- (4) Differentiate with a weak solution of acetic acid (1:1500), controlling the differentiation under the low power of the microscope until the protoplasm of the cells is pink and only the nuclei are blue.
- (5) Wash with distilled water.
- (6) Blot, dehydrate with a few drops of absolute alcohol, clear in xylol and mount in Canada balsam.

Note.—If the eosin tint is too pronounced it can be lightened by the use of very dilute caustic soda solution (1:7000) which is washed off whenever the desired colour has been obtained.

J. H. WRIGHT'S STAIN

This is similar to Leishman's stain and is used more in America than in this country. It should be purchased ready for use. The method of staining is, for all practical purposes, the same as for Leishman's stain.

GIEMSA'S STAIN

This consists of a number of compounds made by adding different proportions of methylene blue and eosin together. These compounds have been designated Azur I, Azur II, and Azur II-eosin. The

preparation is best purchased made up by Grüber or Gurr, but may be prepared as follows :—

Azur II-eosin	3 grams
Azur II	0·8 grams
Glycerin (chemically pure)	250 grams
Methyl alcohol (acetone-free)	250 grams

The stain may be used in a manner similar to Leishman's preparation (the "rapid method"), or prolonged staining may be carried out, as, for example, in staining spirochaetes (the "slow method"). In both cases the preparation must be fixed prior to staining, either by methyl alcohol for three minutes, or by absolute alcohol for fifteen minutes.

RAPID METHOD

- (1) Fix films in methyl alcohol—three minutes.
- (2) Pour on a mixture of 1 part stain and 2 parts distilled water (see p. 168) and allow to act for five minutes.
- (3) Wash with distilled water, allowing the preparation to differentiate for about half-a-minute.
- (4) Blot and allow to dry in the air.

A rapid method with the application of heat is useful for demonstrating spirochaetes :—

Fix preparations with absolute alcohol (fifteen minutes) or by drawing three times through a flame. Prepare a fresh solution of 10 drops of Giemsa's solution with 10 c.c. of distilled water of correct pH (p. 168), shake gently, and cover the fixed film with the diluted stain. Warm till steam rises, allow to cool for fifteen seconds, then pour off and replace by fresh stain and heat again. Repeat the procedure four or five times, wash in distilled water, dry and mount.

SLOW METHOD

A specially valuable method for demonstrating objects difficult to stain in the ordinary way, such as the pathogenic spirochaetes. The principle is to allow the diluted stain to act for a considerable period. As the mixture of stain and water causes a fine precipitate, care has to be taken that this does not deposit on the film.

Cover-slips.—The film is fixed in methyl alcohol for three minutes. A mixture is made in a Petri dish in the proportion of 1 drop of stain to 1 c.c. of distilled water. The cover-slip, when fixed and still wet with the alcohol, is placed carefully, film downwards, on the surface of the mixture. When properly done, the cover-slip remains floating. The lid is carefully placed on the Petri dish and the stain allowed to act overnight. The cover-slip is then washed in a stream of distilled water, allowed to dry in the air and mounted. There should be no deposit of stain precipitated on the preparation.

Slides.—The film is fixed in methyl alcohol for three minutes as with cover-slips. The mixture of stain and distilled water is made in a large (6 in.) Petri dish if there are several slides to stain. A piece of thin glass rod is placed in the Petri dish, and the slides, after fixing, are laid film downwards in the fluid with one end of the slide resting on the glass rod so that there is sufficient staining fluid between the film and the bottom of the dish. After sixteen to twenty-four hours' staining, the slides are washed and dried as in the case of cover-slips.

Adachi's Modification.—This method has been utilised for staining the flagella of *Spirillum minus* (*vide* p. 425) and can also be applied in the staining of delicate spirochaetes. The preparation is fixed for thirty to sixty seconds by osmic-acid vapour over the following solution: osmic acid, 1 gram, distilled water, 100 c.c., 10 drops of 5 per cent. mercuric chloride; and then stained overnight in dilute Giemsa's solution (*vide supra*) to each 10 c.c. of which 0·6 c.c. of 1 per cent. potassium carbonate has been added.

FONTANA'S METHOD OF STAINING SPIROCHAETES

Solutions required—

(a) *Fixative:*

Acetic acid	.	.	.	.	.	1 c.c.
Formalin	.	.	.	.	.	20 c.c.
Distilled water	.	.	.	.	.	100 c.c.

(b) *Mordant* :

Carbolic acid	1 gram
Tannic acid	5 grams
Distilled water	100 c.c.

(c) *Ammoniacal silver nitrate* :

Add 10 per cent. ammonia to 0.25 per cent. solution of silver nitrate in distilled water until the precipitate formed just dissolves. Now add more silver nitrate solution drop by drop until the precipitate returns and does not re-dissolve.

Method—

- (1) Treat the film three times, thirty seconds each time, with the fixative.
- (2) Wash off the fixative with absolute alcohol and allow the alcohol to act for three minutes.
- (3) Drain off the excess of alcohol and carefully burn off the remainder until the film is dry.
- (4) Pour on the mordant, heating till steam rises, and allow it to act for half-a-minute.
- (5) Wash well in distilled water and again dry the slide.
- (6) Treat with ammoniacal silver nitrate, heating till steam rises, for half-a-minute, when the film becomes brown in colour.
- (7) Wash well in distilled water, dry and mount in Canada balsam.

It is essential that the specimen be mounted in balsam under a cover-slip before examination, as some immersion oils cause the film to fade at once.

The spirochaetes are stained brownish black on a brownish-yellow background.

BECKER'S METHOD OF STAINING SPIROCHAETES

Dried, thin, unfixed films should be used. Treat for one minute with :

Glacial acetic acid	1 c.c.
Formalin	20 c.c.
Distilled water	100 c.c.

the fluid being renewed twice during this time. After washing in water (half-a-minute) treat with :

Tannic acid	10 grams
Carbolic acid	1 gram
Distilled water	100 c.c.

for three to five minutes.

After rinsing well in water, place in the following staining solution for three to five minutes :

Basic fuchsin, saturated alcoholic solution	45 c.c.
Shunk's mordant—	
95 per cent. alcohol 16 c.c. }	18 c.c.
Aniline oil 4 c.c. }	
Distilled water	100 c.c.

When making up this stain, thoroughly dry the glass-ware or rinse it well with alcohol. Add the Shunk's mordant to the alcoholic fuchsin solution, mix, and add the distilled water. Filter before use.

Wash well in water, dry, and mount in balsam.

LEVADITI'S METHOD OF STAINING SPIROCHAETES IN TISSUES

(a) ORIGINAL METHOD

1. Fix the tissue, which must be in small thin pieces (1 mm. thick), in 10 per cent. formalin for twenty-four hours.

2. Wash the tissue for one hour in water and thereafter place it in 96-98 per cent. alcohol for twenty-four hours.

3. Transfer to 1.5 per cent. silver nitrate solution in a dark bottle and keep in the incubator for three days.

4. After washing in water for twenty to thirty minutes, place the pieces of tissue in the reducing mixture, made up as follows:—

Pyrogalllic acid	4 grams
Formalin	5 c.c.
Water	100 c.c.

and allow them to remain in this mixture in a dark bottle for forty-eight hours at room temperature.

5. After washing well with water, dehydrate the tissue with increasing strengths of alcohol and embed in paraffin (see p. 179). Thin sections are cut

and mounted in the usual way. After removing the paraffin with xylol the sections are straightway mounted in Canada balsam.

(b) PYRIDIN METHOD

This method is more rapid, but in our experience does not give quite such good results as the older method :

1. Fix the tissue in formalin as in the other method, harden in alcohol overnight and then wash in distilled water.

2. Place the tissue in a 1 per cent. solution of silver nitrate (to which one-tenth of the volume of pure pyridin has been added) for two hours at room temperature, and thereafter at about 50° C. for four to six hours. It is then rapidly washed in 10 per cent. pyridin solution.

3. Transfer to the reducing fluid, which consists of :

Formalin 4 per cent.	.	.	.	.	100 parts
----------------------	---	---	---	---	-----------

to which are added immediately before use :

Acetone (pure)	.	.	.	.	10 parts
Pyridin (pure)	.	.	.	.	15 parts

Keep the tissue in this fluid for two days at room temperature in the dark.

4. After thorough washing, the tissue is dehydrated, embedded, cut and mounted in the usual way.

JAHNEL'S METHOD OF STAINING SPIROCHAETES IN TISSUE

1. Wash in water, for one to three days, thin pieces 2-4 mm. thick, which have been *well fixed* in formalin.
2. Place in pure pyridin for three days.
3. Wash in many changes of water during a period of two to three days until the pyridin almost disappears. Then allow the pieces to remain a few days in 5-10 per cent. formalin.
4. Place in water again for two days.
5. Treat with uranium nitrate (Merck) 1 per cent. solution in distilled water, for half to one hour in the incubator at 37° C. The tissue should rest on lead-free glass-wool to aid penetration.
6. Wash out in distilled water for one day.
7. Allow to remain in 96 per cent. alcohol, three to eight days.
8. Wash out in distilled water until the block sinks.

9. Place the tissue in a dark flask and treat it with a freshly prepared silver nitrate solution 1.5 per cent., for five to eight days in the incubator at 37° C.
10. Decant the silver nitrate solution, wash the tissue in water, and transfer to the following developer for one to two days :

Pyrogallol, 4 per cent.	. 90 c.c.	} of this reject 15 c.c. and replace by pyridin.
Acetone	. 10 c.c.	
11. Wash in distilled water ; embed in paraffin in the usual manner.

WARTHIN AND STARRY'S SILVER-AGAR METHOD OF STAINING SPIROCHAETES IN FILMS

(1) Make film on thoroughly clean No. 1 cover-glass and dry carefully in air. (2) Place in absolute alcohol for three to five minutes. (3) Wash in distilled water. (4) To clear the background place in concentrated hydrogen peroxide for fifteen minutes and wash in distilled water. (5) Rinse the preparation in 2 per cent. silver nitrate and then cover the film with a second clean cover-glass which has also been rinsed in the silver nitrate solution. (6) Place the two glasses adhering together in 2 per cent. silver nitrate and keep in the incubator for one to two hours, then remove and separate them. (7) Place cover-glass with film uppermost in the following mixture : 2 per cent. silver nitrate, 3 c.c. ; 10 per cent. aqueous gelatin (warm), 5 c.c. ; glycerol (warm), 5 c.c. ; 1.5 per cent. agar suspension (warm), 5 c.c. ; 5 per cent. aqueous hydroquinone, 2 c.c. (8) After the solution is reduced remove preparation and rinse in 5 per cent. sodium thiosulphate. (9) Wash in distilled water. (10) Treat with absolute alcohol, clear with xylol and mount in balsam.

This method may also be applied in the staining of sections mounted on cover-glasses.

STAINING OF AMOEBAE AND OTHER INTESTINAL PROTOZOA IN FAECES

Wet smears should be fixed in a mixture of

Alcohol	1 part
Saturated aqueous solution of corrosive sublimite	2 parts

for five minutes or longer.

The films are then washed in 50 per cent. spirit and treated with Gram's iodine for two minutes, to remove the mercury salt, the iodine being removed with spirit and the films washed.

Stain with iron haematoxylin for ten to twenty minutes.

Iron Haematoxylin :

(a) Haematoxylin	1 gram
Absolute alcohol	100 c.c.
(b) Liquor ferri perchlor. 30 per cent.	4 c.c.
Concentrated hydrochloric acid	1 c.c.
Distilled water	100 c.c.

Mix equal parts of (a) and (b) immediately before using.

After staining, wash films in water, pass through alcohol, clear with xylol and mount in balsam, as in the treatment of tissue sections.

Preparations may be counter-stained with van Gieson's stain, fifteen to thirty seconds :

Saturated aqueous solution of acid fuchsin	1-3 parts
Saturated aqueous solution of picric acid	100 parts

Dehydrate rapidly with absolute alcohol, clear in xylol and mount in balsam.

Note.—Fixed wet preparations must be treated in the same manner as sections and never allowed to become dry.

STAINS FOR RICKETTSIA, AND INCLUSION BODIES IN VIRUS INFECTIONS

Castaneda's Stain for Rickettsia Bodies ¹

A buffer formaldehyde solution is prepared as follows : (a) 23·86 grams di-sodium phosphate are dissolved in 1 litre of distilled water ; (b) 11·34 grams of monopotassium phosphate are dissolved in 1 litre of distilled water. 82 parts of (a) are mixed with 12 parts of (b) and the mixture is filtered through a Berkefeld candle or Seitz filter. To this is added 0·2 per cent. of pure formalin as a preservative. The final pH before the formalin is added should be pH 7·6.

20 c.c. of buffer solution are mixed with 1 c.c. formalin, and 3 drops of Löffler's methylene blue or 10 drops of a 1 per cent. aqueous methylene blue. Staining is continued for two or three minutes. The preparation is then washed in running water for thirty seconds. Counter-stain with an 0·1 per cent. aqueous solution of safranin for one or two seconds. The Rickettsia bodies remain blue, while the protoplasm and nuclei of the cells are red.

This stain is also useful for "elementary bodies"—e.g. those of psittacosis. It may be modified by using Azur II in place of the Löffler's methylene blue.

¹ M. R. Castaneda, *Journ. Infect. Diseases*, 1930, vol. 47, p. 416.

Staining of Inclusion Bodies

For intranuclear and cytoplasmic inclusions Giemsa's stain (p. 169) is the most generally useful.

A good stain also is phloxine 2.5 per cent. aqueous solution followed by borax methylene blue. Sections should be stained with phloxine for one hour, preferably at 37° C., washed in water and stained with borax methylene blue for two hours at room temperature. Decolorise in 75 per cent. alcohol, dehydrate in absolute alcohol and mount in balsam.

FIXATION AND EMBEDDING OF TISSUES; SECTION CUTTING

As the ordinary routine bacteriological investigation of tissues is carried out almost exclusively with paraffin sections, this technique only will be described.

The fixed tissue is embedded in paraffin wax to support it during the cutting of the section, and the section is held together by the wax in the process of transferring it to the slide.

The paraffin wax must completely permeate the tissue, but before it can do so, all water must be removed from the material and replaced by a fluid with which melted paraffin will mix.

Water, therefore, is first removed by several changes of alcohol; the alcohol is replaced by some fluid—such as benzol, xylol, acetone, chloroform—which is a solvent of both alcohol and paraffin wax, and the tissue is finally embedded in melted paraffin.

Before removing the water from the tissue preparatory to embedding, the tissue must be suitably fixed and hardened.

The essentials for obtaining good sections are :

- (1) The tissue must be perfectly fresh.
- (2) It must be properly fixed by using small pieces and employing a large amount of fixing fluid.
- (3) The appropriate fixing fluid must be employed for the particular investigation required.
- (4) The tissue must not remain too long in the embedding bath.

FIXATIVES**FORMALIN**

Ten per cent. commercial formalin in normal saline solution is a good fixative for general use. Its advantages are: it is easily prepared, has good penetrating qualities, does not shrink the tissues, and permits considerable latitude in the time during which specimens may be left in it. Moreover the subsequent handling of the material is much easier in our experience than in the case of corrosive sublimate fixatives, such as Zenker's fluid. Formalin fixation is not so good as other methods where fine detail has to be observed, as, for example, in material containing protozoa. For general routine use, however, it is the most convenient and useful of fixatives. Tissue should be cut into thin slices, about $\frac{1}{8}$ -in. thick, and dropped into a large bulk of fixative. The fluid may be changed at the end of twenty-four hours, and fixation is usually complete in forty-eight hours. Specimens are then washed in running water for an hour and transferred to 50 per cent. spirit. In the latter fluid they may be kept for a considerable time without deterioration.

ZENKER'S FLUID

Corrosive sublimate	.	.	.	.	5 grams
Potassium bichromate	.	.	.	.	2.5 grams
Sodium sulphate	.	.	.	.	1 gram
Water	.	.	.	.	100 c.c.

Immediately before use, 5 c.c. of glacial acetic acid per 100 c.c. of fluid are added.

The fluid should be warmed to body temperature and only small pieces of tissue must be placed in it. Fixation is complete in twenty-four hours, and thereafter the pieces of tissue are washed in running water for twenty-four hours to remove the potassium bichromate and corrosive sublimate. The tissue is then transferred to 50 per cent. spirit.

It is essential that all corrosive sublimate should be removed, otherwise a deposit will appear in the sections. The bulk of the corrosive sublimate is removed by washing. The remainder can be removed with iodine during the dehydration stage in alcohol. The material after washing is transferred to 50 per

cent., and later to 70 per cent. alcohol to which sufficient iodine has been added to make the fluid dark brown in colour. (It is convenient to keep a saturated solution of iodine in 90 per cent. alcohol in a drop bottle, and add a few drops as required.) If the alcohol becomes clear more iodine is added until the fluid remains brown. This indicates that all the corrosive sublimate has been dissolved out by the iodine-alcohol.

Cut sections fixed on slides can also be treated with iodine—*e.g.* Gram's iodine—for three to five minutes, to remove mercuric chloride.

Animal tissues fixed in Zenker's fluid are more difficult to cut, and sections are apt to float off the slide, particularly if fixation has been unduly prolonged.

ZENKER-FORMAL FLUID

This is similar to Zenker's fluid except that the acetic acid is omitted and 5 c.c. of strong formalin are added per 100 c.c. immediately before use. It is a useful general fixative for animal tissues.

MERCURIC CHLORIDE—FORMALIN SOLUTION

Mercuric chloride saturated aqueous solution	90 c.c.
Formalin, commercial	10 c.c.

Small portions of tissue must be used and fixation is complete in one to twelve hours. Then transfer to alcohol and iodine as after Zenker's fluid (*q.v.*). This fluid fixes with the minimum amount of distortion and the finer cytological details of the cells are retained.

EMBEDDING AND SECTION CUTTING

After fixation by any of the previous methods and transference to 50 per cent. alcohol, *small pieces* of tissue are treated as follows :—

- (1) Place in 90 per cent. spirit for two to five hours.
- (2) Transfer to absolute alcohol for two hours.
- (3) Complete dehydration in fresh absolute alcohol for two hours.
- (4) Transfer to a mixture of absolute alcohol and chloroform (equal parts) till tissue sinks, or overnight.
- (5) Place in pure chloroform for six hours.

- (6) Transfer the tissue for one hour to a mixture of equal parts of chloroform and paraffin wax, which is kept melted in the paraffin oven.
- (7) Place in pure melted paraffin in the oven at 55° C. for two hours, preferably in a vacuum embedding oven.

The tissue is embedded in blocks of paraffin. These are cut out, trimmed with a knife, and sections 4 μ thick are cut by means of a microtome. The sections are flattened on warm water, floated on to slides and allowed to dry. Albuminised slides are useful where the staining process involves heating, and where animal tissue is used, especially after fixation with Zenker's fluid. The slides are coated with albumin either by means of a small piece of chamois leather or by the finger. The albumin solution is made by adding three parts of distilled water to one part of egg-white and shaking thoroughly. The mixture is filtered through muslin into a bottle, and a crystal of thymol is added as a preservative. It is usual to coat a number of slides, and, after drying, the slides are stored until required. The albuminised side may be identified by breathing gently on the slide; it is not dimmed by the breath, whereas the plain side is.

For further treatment of sections, see Staining Methods.

For additional details, reference must be made to works on histology.

CHAPTER VI

ANIMAL INOCULATION AND AUTOPSY

IN Great Britain animal experiments may be performed only under a licence granted by the Home Secretary. In addition to the licence various certificates have also to be obtained, depending on the nature of the experiments and the animals used.

The usual animals employed for bacteriological experiments are the guinea-pig, rabbit, mouse and white rat, and *the commonest method of inoculation is by means of a hypodermic needle and syringe*. According to the method of inoculation and the size of the animal, the amount of injected material varies, and a number of syringes of different sizes are used. A convenient "battery" of syringes is the following:—1 c.c. "tuberculin" syringe graduated in 0·01 c.c. ; 2 c.c. syringe graduated in 0·1 c.c. ; 5 c.c. graduated in 0·25 c.c. ; 10 c.c. graduated in 0·5 c.c. A 20 c.c. syringe is used only occasionally. Syringes may be of the "Record" type, or of the all-glass "Luer" type made of pyrex glass. A selection of needles is required, of which the following are useful:—fine-bore, No. 25 gauge ; medium-bore for general use, No. 21 or 22 gauge ; large-bore, Nos. 16–18 gauge, for inoculating thick suspensions or emulsions of tissue.

Syringes are sterilised by taking them apart and boiling for five to ten minutes. All-glass syringes may conveniently be sterilised ready for use as follows. The needle is fitted and the plunger lightly smeared with liquid paraffin and inserted into the barrel. The assembled syringe is placed in a test-tube longer than

itself, and of such a bore that the shoulder of the barrel rests on the rim. The shoulder is wrapped with a little gauze or kraft paper so that it does not rest directly on the glass. The whole is wrapped in kraft paper and sterilised in the hot-air oven.¹ The syringe remains sterile indefinitely in its wrapping and is always ready for immediate use. After use it should be washed out with weak lysol. If a "Record" syringe, it is again boiled, then dried and put away with the piston and barrel separate, or else a small quantity of liquid paraffin is smeared over the plunger before it is fitted into the barrel. If an all-glass syringe, it is washed with water, then wiped with methylated spirit, and finally coated over with liquid paraffin. It is then put up in a test-tube, as above described, and sterilised. Needles may be stored in alcohol or rust-preventing fluid,² or dried and a little alcohol run through them. The stilette should be lightly smeared with vaseline before replacing it in the needle. Needles should always be kept sharp and the points renewed on a fine oil-stone (Arkansas slip-stone). Needles made from stainless steel are recommended for use, as they do not rust.

MATERIAL INOCULATED

Urine, cerebro-spinal and other *fluids* and *blood* are easily inoculated with a medium-bore needle. Tenacious material such as *pus* and *sputum* are injected by means of a wide-bore needle.

Cultures.—Fluid cultures are easily drawn through a medium-bore needle. It will be found advantageous first to pour the culture into a small (2 in.) Petri dish, or a 50 c.c. conical test-glass. Growths on solid media

¹ "Record" syringes must never be sterilised in the hot-air oven, as the heat will melt the cement or solder which unites the glass barrel with the metal parts.

² Potassium bichromate	4 grams
N/1 NaOH	4 c.c.
Distilled water	to 100 c.c.

may be scraped off and emulsified in broth or saline, or the diluting fluid may be poured on the culture, which is then emulsified with a wire loop.

Tissues.—These should be cut into small pieces in a sterile porcelain mortar by means of scissors sterilised by boiling. Some clean coarse sand, contained in a stoppered bottle, and sterilised by hot air, is then added to the mortar and the whole thoroughly ground with the pestle. When the tissue has been well ground up, saline is added, and the mixture further triturated. On standing for a short time, the sand and tissue rapidly settle to the bottom of the mortar, and the supernatant fluid can be drawn into the syringe. When intravenous inoculation of tissue emulsion has to be employed, care must be taken that no large particles are injected. To avoid this, the emulsion must be centrifuged at low speed, and only the supernatant fluid used.

GUINEA-PIGS

These animals vary in size, and weigh from 200 grams (small) to 1000 grams (large). A good average weight for general purposes is 400 grams.

METHODS OF INOCULATION

Subcutaneous.—An assistant holds the animal during the operation, and the injection is made under the skin of the flank. The animal is grasped across the shoulders in one hand, with the thumb curved round the animal's neck so that it rests on the lower jaw. The hind legs are secured between the first and second, and second and third, fingers of the other hand, the knuckles being uppermost, and the animal is held so that the flank is presented for inoculation. The skin may be disinfected with tincture of iodine, but it is not necessary to do so. The operator picks up a fold of skin and introduces the point of the needle into the base of the fold, so that it lies in the subcutaneous tissue. Amounts up to 5 c.c. can be

introduced. A 2 c.c. or a 5 c.c. syringe is convenient for the purpose.

Intraperitoneal.—The animal is held in a similar manner. The inoculation is made in the mid-line in the lower half of the abdomen. The skin may be sterilised. The assistant holds the animal with its head downwards, so that the intestines fall towards the diaphragm. The skin is pinched up, the point of the needle passed into the subcutaneous tissue, and then downwards through the abdominal wall into the peritoneal cavity. There is no risk of damage to the intestines. Not more than 5 c.c. can safely be inoculated intraperitoneally.

Intracutaneous.—This method is used chiefly in testing cultures of the diphtheria bacillus for virulence (*vide* p. 286). The hair is removed from the flanks of the animal by means of a depilating powder or a fresh 5 per cent. solution of sodium sulphide. White guinea-pigs (300–400 grams weight) are used, as the skin is unpigmented and the results of the test can easily be read.

The depilating powder is made as follows :

Barium sulphide, commercial powder	.	7 parts
White household flour		7 parts
Talcum powder		7 parts
Castile soap powder		1 part

Remove the hair from the flanks as closely as possible with hair clippers. Make up the depilating powder into a smooth paste with water, and rub into the animal's hair with a wooden spatula or toothbrush. Allow the paste to act for one minute and renew the application. After two minutes remove the paste with the spatula or handle of the toothbrush. Now wash the animal's skin and surrounding hair with warm water and dry with a cloth. The depilated surface should be quite smooth and white, and it is not advisable to leave the paste on too long as the skin becomes red and excoriated in patches, making the subsequent reading of reactions very difficult. The

depilating powder should be used at least one hour before the intracutaneous injection is carried out.

For the test a 1 c.c. all-glass tuberculin syringe, fitted with a short needle of 25 or 26 S.W.G. (exactly as used for Schick and Dick tests), is employed. The skin of the animal is pinched up between the thumb and forefinger, and the point of the needle is inserted at the top of the fold so that the bevel of the needle is towards the surface of the skin. The needle passes only into the dermis, as near the surface as possible, and not into the subcutaneous tissue. 0.2 c.c. is the amount usually used, and when several tests are to be made the injections should be about one inch apart and not too near the middle line of the abdomen. No more than ten injections should be made on one animal. The results are read twenty-four to forty-eight hours later.

The normal temperature of the guinea-pig is 101.5° F. To ascertain the animal's temperature, a clinical thermometer, with a small round bulb which is smeared with vaseline, is gently inserted into the rectum of the animal.

RABBITS

These animals are often unsatisfactory for experimental purposes owing to their liability to parasitic and intercurrent infections. The animals used should be free from snuffles (a chronic nasal inflammation), subcutaneous abscesses, and mange. They should be plump, their fur should be in good condition, and they should not be suffering from diarrhoea. If the animal is in poor condition it is probably affected with coccidiosis or intestinal worms. Rabbits are very prone to die from septicaemia (due to *B. leptisepticus*), and from pneumonia. It is better to use animals obtained from a reliable breeder, where the condition of the stock is known, than to purchase rabbits casually and indiscriminately from a dealer.

The normal rectal temperature of the rabbit is 102.4° F., but the variations are great. No temperature under 104° F. should be considered pathological.

The leucocyte count of the rabbit is subject to great normal variation.

The chief use of the rabbit lies not so much in diagnostic work as in its value for experimental purposes. It is extensively used for the production of immune sera, such as agglutinating and haemolytic sera, which are frequently employed for routine laboratory diagnosis.

Under Certificate "A" of the Home Office the animal may be inoculated intravenously, intraperitoneally, subcutaneously or by scarification, without the use of an anaesthetic.

Scarification.—The hair is removed from the flank of the animal by first clipping and then shaving, or by means of the depilating mixture described on p. 184. The skin is cleansed with alcohol, which is allowed to evaporate. A number of parallel scratches are made with a sharp sterile scalpel, just sufficiently deep to draw blood. The infective material is rubbed into the scarified area by means of a platinum loop or the side of the scalpel. This method is mainly used for the propagation of vaccinia virus.

Intravenous inoculation is employed when material has to be introduced directly into the circulation. The marginal vein of the ear is the most convenient site. The rabbit may be held by an assistant or placed in a special box so that only its head protrudes. The hair over the vein should be dry-shaved with a sharp razor. The vein may be distended for ease of inoculation either by vigorous rubbing with a piece of cotton-wool or by holding the ear over an electric-light globe, when the heat causes a dilatation of the blood vessels. According to the amount of material to be injected, a suitable syringe furnished with a needle is selected and sterilised by boiling or other means. It is a mistake to use too fine a needle. The operator faces the animal and the ear is held horizontal by means of the left hand. The needle is kept as nearly parallel as possible to the vein and the point inserted towards the head of the animal. When the injection is completed, the

needle is withdrawn, and a small piece of cotton-wool placed on the vein, which is then compressed between the thumb and finger.

Note.—In removing samples of blood from the rabbit a similar procedure is adopted. The ear is shaved, sterilised with alcohol and painted with sterile vaseline. The vein is dilated by gently rubbing with benzol or xylol; an incision is made into it by means of a large triangular needle or a sharp scalpel. The blood is then allowed to drop into a test-tube. When sufficient blood has been removed a small piece of cotton-wool is pressed firmly over the incision in the vein. The ear is finally washed with spirit and lightly smeared with vaseline (*vide* p. 106).

Intraperitoneal inoculation is carried out as in the case of the guinea-pig.

Subcutaneous inoculation may be made either into the abdominal wall, or into the loose tissue about the flank or at the back of the neck. The hair is clipped, the skin is sterilised with iodine and then pinched up, and the needle is inserted. The technique is the same as that for the guinea-pig.

Cardiac puncture.—*Vide* p. 107.

Intracerebral inoculation.—*Vide* p. 469.

RATS

Care must be exercised in handling these animals, as the sharp incisor teeth are capable of inflicting a severe wound. They should be held by the loose tissue at the nape of the neck with a pair of crucible tongs or artery forceps, and the animal is kept taut by pulling on the tail. Intraperitoneal and subcutaneous injections are made in a manner similar to that used for the guinea-pig.

Intravenous inoculation may be made into the vein at the root of the tail. The vein should be dilated by immersing the tail in warm water.

MICE

Subcutaneous inoculation.—An assistant grasps the loose skin at the nape of the neck in one hand and

the tail in the other. In this manner the animal is held in a fixed position while the needle is introduced under the skin near the root of the tail. Amounts up to 1 c.c. may be injected.

Intraperitoneal inoculation may be carried out if the animal is similarly held and then turned over. For steadiness, the assistant's arms should rest on the table. The injection is made to one side of the middle line in the lower half of the abdomen and amounts up to 2 c.c. can be introduced.

Intraperitoneal inoculation may also be done without an assistant. The animal is held at the nape of the neck with the left hand, and kept extended by holding the tail with the right hand. The left hand is turned over so that the mouse lies on its back in the upturned palm. The tail is then fixed by the little finger of the left hand. The mouse is now firmly held, and the right hand is free to pick up the syringe and make the injection.

Intravenous inoculation may be made into a vein at the root of the tail if a fine needle be used and the vein dilated by placing the tail of the animal in warm water.

A small cylindrical cage made of perforated zinc, and just large enough to hold the mouse with its tail protruding, is useful for this procedure.

Intracerebral Inoculation.—The skin over the head is depilated (*vide* p. 184) under slight ether anæsthesia. The animal is completely anæsthetised for the inoculation and the depilated area sterilised with iodine-spirit. A fine-bore needle attached to a 1 c.c. syringe (as used for intradermal inoculation—*vide* p. 185) is employed and easily penetrates the skull. The site of injection is just posterior and lateral to the vertex and the point of the needle is carried through the skull for $\frac{1}{8}$ in. to $\frac{1}{4}$ in. Approximately 0.05 c.c. of fluid can be injected with safety.

AUTOPSY

All experimental animals, whatever the cause of death, should be examined post-mortem as a routine. When a virulent organism such as the bacillus of plague or of anthrax has been used, especial care must

be taken, otherwise the infection may be disseminated, with danger to the operator and other workers.

Details will be given of the procedure in conducting an autopsy in the usual manner; and also the method used when dealing with highly infectious organisms.

As the primary reason for the autopsy is to recover organisms previously injected into the animal in addition to observing the lesions, the examination must be conducted with strict aseptic precautions :

Materials required :

A suitable animal board, on which the carcass can be fixed in the supine position.

Instruments.—Three scalpels ; scissors, ordinary size, four pairs ; mouse-toothed forceps, four pairs ; small bone forceps if the skull is to be opened ; a searing iron—a 4 oz. soldering bolt is excellent for the purpose ; sterile capillary pipettes ; sterile Petri dishes ; sterile test-tubes, and tubes of media.

The knives are sterilised in strong lysol, and the metal instruments by boiling in a sterilising bath—*e.g.* an enamelled “ fish-kettle.” When ready for use, the tray of instruments is lifted out of the steriliser and laid on a spread towel which has previously been soaked in 1 : 1000 solution of perchloride of mercury.

It is our practice, where cultures have to be made, first to immerse the animal completely in weak lysol solution (3 per cent.) for ten minutes. This not only destroys most of the surface organisms, but prevents the dust in the fur from getting into the air and contaminating other materials. The animal is now fixed to the board, and towels moistened with antiseptic are placed over the head and lower extremities.

The instruments are removed from the steriliser. A long median incision through the skin of the abdomen and chest is now made, and the skin widely dissected, exposing the abdominal and chest muscles. With another set of instruments the peritoneal cavity is opened, and the abdominal wall is reflected to each side. With fresh instruments remove the spleen and

place it in a sterile Petri dish. Other organs such as the liver and kidneys may be similarly removed. The ensiform cartilage is now tightly gripped with a pair of strong forceps, and by means of a sterile pair of strong scissors a cut is made on either side of the chest through the costal cartilages. The sternum is raised and pulled towards the head. The heart is now exposed. A sterile capillary pipette, furnished with a teat, is passed through the heart wall. Blood can thus be withdrawn and inoculated into various media. If the autopsy has been properly performed, it is not necessary to sear the surface of the heart. The lungs are then removed, using fresh instruments, by cutting each organ free at the hilum. Care must be taken not to open into the oesophagus if the lungs are to be used for cultural purposes.

After the organs to be used for culture have been removed and placed in separate Petri dishes, the autopsy can be completed.

While the instruments are again being boiled the naked-eye appearances of the organs should be studied. For culture, the spleen gives the best results, but the other solid viscera may be similarly used. The organ is cut with sterile instruments and a small portion is taken up with a stiff wire and smeared on the surface of solid media. Liquid media are inoculated with a small fragment of the tissue.

In conducting post-mortem examinations, various animal diseases, such as worm infestation, coccidiosis, pseudo-tuberculosis, etc., may be noticed, and the worker should be familiar with their appearances.

When the animal is infected with highly pathogenic organisms the worker *must* wear rubber gloves. The animal is soaked in antiseptic solution as before, and nailed to a rough piece of board of the appropriate size. This board is then placed in a large enamelled iron tray. The autopsy is carefully performed in the usual way. The carcase is finally covered with 10 per cent. lysol, which flows over the board and into the tray. The whole contents of the tray—board and

carcase—are then destroyed in a furnace. The rubber gloves, instruments and tray are thoroughly sterilised. When performing animal autopsies we strongly advise the wearing of a large overall made of waterproof material, and, in addition, the use of some form of glasses or goggles to protect the eyes.

CARE OF ANIMALS¹

General Directions

Litter for all animals may be sawdust, wood chips, or peat moss.

All cages and runs should be cleaned out twice weekly. The daily duties of the animal-keeper should be written out and posted up in the animal-house. Feeding-troughs should be of earthenware, about four inches in diameter. They must be cleaned daily, and in the case of experimental animals it is important that the troughs are returned to their respective cages.

GUINEA-PIGS

Guinea-pigs should be fed twice daily.

Morning—

Oats, 1 part	.	.	.	.	} 3 oz. per animal
Bran, 3 parts	.	.	.	.	
Water (for stock in runs)					

Afternoon—

Hay	.	.	.	.	2 oz.
Cabbage or roots	.	.	.	.	3-4 oz.
Water as above					

Cages.—Stock runs should be about 3 ft. square, with sides 1 ft. 8 in. high.

For experimental animals, galvanised-iron cages are best, as they are more easily cleaned and sterilised after use. A convenient size is $13\frac{1}{2} \times 8\frac{1}{2} \times 6\frac{1}{2}$ in., fitting in a loose tray $14\frac{1}{2} \times 9\frac{1}{2} \times 1\frac{3}{4}$ in.

Breeding.—Period of gestation seventy days; animals may be used for breeding after six months; three litters yearly, average litter three; young weaned at fourteen days.

¹ For full details of the care of laboratory animals, reference should be made to *A System of Bacteriology*, Medical Research Council, 1931, vol. ix., chap. 17, from which certain of the data in this section are quoted by permission of the Controller, H.M. Stationery Office.

Common Diseases

Pseudo-tuberculosis (vide p. 370).—May be either acute or, more commonly, chronic. In the acute type the animal dies in a few days. In the chronic type the liver, spleen and mesenteric glands show very numerous yellowish-white areas scattered through them, somewhat suggestive of tuberculosis. Often a whole stock becomes infected, and experimental animals frequently die before the experiment is completed.

Respiratory Infections.—Guinea-pigs are liable to pneumonia and pleurisy, often with haemorrhage and septicaemia due to such organisms as the pneumococcus, *B. of Friedländer*, *Pasteurella* group, etc.

Intestinal Infections.—Organisms of the *Salmonella* group are often responsible, and epizootics are liable to occur in overcrowded stock.

Virus diseases, such as guinea-pig paralysis, may be met with.

RABBITS

Feeding, per animal

Morning —

Oats, 1 part	.	.	.	.	.	} 4-5 oz.
Bran, 3 parts	.	.	.	.	.	
Water						

Afternoon—

Hay	.	.	.	.	.	4-5 oz.
Cabbage or roots	.	.	.	.	.	5-6 oz.
Water						

Cages.—Galvanised iron or zinc should be used. A convenient size is $19\frac{1}{2} \times 14 \times 14$ in., with a door in front, resting in a loose tray $20\frac{1}{2} \times 15 \times 2$ in. Young rabbits up to three months of age may be housed together, after that time the sexes should be separated.

Breeding.—Period of gestation thirty-one days; animals may be used for breeding after six months; four litters yearly; average litter four; young weaned at six weeks. The mother should be disturbed as little as possible for the first ten days after parturition.

Common Diseases

Pseudo-tuberculosis (vide p. 370).—This is a chronic disease, in which the animal loses weight and eventually dies. The liver and spleen particularly show numerous well-defined yellow areas like miliary tubercles.

Respiratory Tract Infections—of which *Snuffles*, due to *B. lepi-septicus*, is the most common. Infected animals should be destroyed at once.

Intestinal Infections.—Diarrhoea may be due to organisms of the *Salmonella* group—e.g. *B. aertrycke*. It is also sometimes caused by *coccidiosis*.

Parasitic Mange.—This usually affects the ears, and is best treated by liquid paraffin containing 1 per cent. of phenol.

Worms.—The cysticercus stage of the dog tape-worm, *Taenia pisiformis*, is the commonest type of infestation, and is characterised by numerous cysts in the omentum, and occasionally in the liver.

Ulcerative lesions of the genitals due to *Treponema cuniculi* (which is very similar morphologically to *Treponema pallidum*, q.v.) are frequently seen.

RATS

Feed only once daily, preferably in the afternoon. Give mash of boiled meal, mixed with fresh oats and bran, and made into balls the size of a walnut. An adult rat requires 2 oz. of this daily. Also give fresh cabbage in the mixture three times weekly, and some biscuit and cod-liver oil once a week. Rat food should be given immediately it is made, and not allowed to remain over till the next day.

Drinking water from a special glass container should always be available.

Cages.—Galvanised-iron cages with narrow mesh are used for experimental animals.

For stock animals large wooden boxes with zinc bottom and perforated zinc top, 36 × 18 × 15 in., are suitable.

Breeding.—Period of gestation three weeks; three to four litters yearly; average litter six; young are weaned at six weeks, and the female rested a further two weeks.

Common Diseases

Rats suffer rarely from intercurrent infections, except mange.

Respiratory Infections—e.g. pneumonia—may occur, but not frequently.

Intestinal Infections.—*Salmonella* infections—e.g. *B. enteritidis*—may cause fatal epizootics.

Mange appears especially at the root of the tail and ears, as a brown scaly condition. The animals should be examined twice weekly, and if infected the parts should be smeared with an ointment composed of flowers of sulphur, 2 parts; sodium carbonate (anhyd.), 1 part; lard or vaseline, 16 parts.

MICE

Feeding.—Feed only *once* daily, preferably in the afternoon, with stale dry bread soaked in water, and squeezed and made up in pieces the size of a walnut, or alternatively with a mixture of oats 1 part, and bran 3 parts, moistened and made up into small balls as above; allow one per animal. A little cod-liver oil should be placed in the mash once weekly. A pinch of canary seed and millet seed should be allowed each animal weekly. Drinking water is essential and should be provided from a special glass container.

Cages.—Stock cages for breeding, etc., are made of wood with a zinc bottom. The lid is of perforated zinc with wooden edges. The size is $24 \times 12 \times 12$ in.

For individual mice in a one- or two-day experiment—*e.g.* pneumococcus inoculation—a 1 lb. or 2 lb. screw-capped preserving jar, with a piece of perforated zinc in place of the glass portion of the lid, is most convenient.

Breeding. Mice are easily bred. Place one male and two to five females in the box. Remove male after fourteen days and the females will litter together. Period of gestation eighteen to twenty-one days; four litters yearly; average litter four. Young are weaned at one month; female is rested a further two weeks. It is advisable to replace breeding stock after three litters. An increase of millet and canary seed should be given to breeding animals.

Common Diseases

Intestinal infections due to organisms of the *Salmonella* group (*B. enteritidis* and *B. aertrycke*), and termed "Mouse typhoid," may produce severe epizootics. Existing stock should be destroyed, boxes disinfected and fresh stock obtained.

Ringworm is frequently met with.

Worms may occasionally cause ill-health or death.

Tumours are frequent, particularly mammary carcinoma.

CHAPTER VII

IMMUNOLOGICAL & SEROLOGICAL METHODS AS APPLIED TO BACTERIOLOGY¹

THE WIDAL REACTION AND OTHER AGGLUTINATION TESTS

THE nature of the *Widal reaction* and its applications are referred to on pp. 31, 33, 335.

DIRECTIONS FOR OBTAINING SPECIMEN OF BLOOD SERUM FOR THE TEST

The blood specimen should be taken by vein puncture² (*vide* p. 141), so as to obtain a satisfactory amount of serum for detailed examination, especially when the reactions with the paratyphoid bacilli as well as *B. typhosus* require to be tested. At least 5 c.c. of blood should be obtained, and the blood immediately transferred from the syringe to a stoppered sterile tube and allowed to clot. When the serum has separated, it is pipetted off into a sterile tube.

A small amount of serum may, however, suffice for the Widal test, and can be obtained by the following method if it is preferred to vein puncture.

¹ Only those immunological and serological methods which are applicable to routine bacteriological work are dealt with. For other and special methods, and the methods of preparing and testing therapeutic antisera, reference should be made to larger works on bacteriology and immunity.

² In general medical practice the Behring Venule is a most convenient instrument for obtaining a blood sample from a vein (*vide* p. 145).

The *requisites* are : triangular needle, small glass-cutting file, spirit lamp or Bunsen burner, gauze or cotton-wool, methylated spirit, and a Wright's blood capsule (1½-2 in. of glass "quill" tube, 5 mm. bore, drawn out at the ends to capillary dimensions and sealed, with one of the capillary limbs bent in a semicircle).

The blood is drawn by puncturing the skin of the finger near the base of the nail. Sterilise the needle and the patient's finger with spirit. Break off the tips of both ends of the capsule, notching first with the file. Puncture the skin by a deep thrust of the needle. When a large drop of blood collects, dip the end of the curved limb of the capsule in it and allow the blood to pass into the capsule by capillary action. The capsule should, if possible, be filled three-quarters full. If blood does not flow freely, pressure may be applied by winding a narrow bandage round the base of the finger. A second puncture may facilitate the filling of the capsule. The success of the method depends on obtaining a *continuous* free flow of blood. If there is any delay and the blood coagulates in the capillary part of the capsule before the required amount is collected, another capsule must be used. To close the capsule, heat the straight end in the flame and seal off the tip. As this end cools, the blood is retracted from the bent limb, which may then be sealed without heating or charring the blood. Both ends must be *completely* sealed.

After the blood coagulates in the capsule, the serum can be separated by centrifugalisation, the bent limb serving to hang the capsule on the rim of the centrifuge tube. A hand centrifuge is sufficient for this purpose. The capsule is opened by filing and breaking it at the bent end, and the serum is drawn off by means of a capillary pipette.

Some workers prefer a blood capsule of smaller calibre and with both capillary ends straight.

THE AGGLUTINATION TEST

Requisites :

1 c.c. pipette graduated to the tip in 1/10ths and 1/100ths ; 0.1 c.c. pipette graduated to the tip in 1/100ths and 1/500ths ; a rubber teat, or preferably a mouth-piece for pipetting by suction—*i.e.* 3 in. of 5 mm. bore quill tube with 9-12 in. of rubber tubing attached which can be fitted to the top of the pipette (*vide infra*). The free end of the mouth-piece is "smoothed" in the Bunsen flame.

Steriliser with boiling distilled water for pipettes, etc. (*vide p. 69*) ; sterile 0.85 per cent. saline ; test-tubes $3 \times \frac{1}{2}$ in. ; agglutination tubes $3 \times \frac{1}{8}$ in. ; test-tube racks suitable for the tubes ; small beaker or conical test-glass for saline solution ; grease pencil for marking tubes ; capillary pipette.

Bacterial Suspension

Prepare a suspension of *B. typhosus* by adding in fractions 5 c.c. of saline to a well-grown twenty-four-hours' agar slope culture, and emulsifying the growth with the aid of a platinum loop. This emulsion can be standardised to a suitable opacity—e.g. tube 5, Brown's opacity standards (*vide* p. 228). The emulsion is decanted and allowed to stand for half-an-hour until bacterial clumps and fragments of agar have sedimented. Alternatively, the emulsion may be centrifuged for one minute.

Serum Dilutions

First make up a 1 in 15 dilution of the patient's serum, and from this prepare a series of doubling dilutions in the small ($3 \times \frac{1}{2}$ in.) test-tubes :

①	②	③	④	⑤	⑥	⑦
1 in 15	1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	CONTROL— NO SERUM

The actual procedure is as follows :—

In a small test-tube add 0.1 c.c. patient's serum to 1.4 c.c. saline—i.e. a 1 in 15 dilution.

Wash out the pipette thoroughly in saline solution.

In a metal rack place seven small ($3 \times \frac{1}{2}$ in.) tubes as above.

Add 0.4 c.c. saline to each of the tubes (2) to (7). Add to tubes (1) and (2) 0.4 c.c. of the 1 in 15 dilution of patient's serum. The dilution of serum in tube (2) is now 1 in 30. Withdraw 0.4 c.c. from tube (2) into tube (3), making now in tube (3) a 1 in 60 dilution of serum. Withdraw 0.4 c.c. from tube (3) into tube (4)—i.e. 1 in 120—and so on till a dilution of 1 in 480 is obtained in tube (6). Withdraw 0.4 c.c. from tube (6) and discard. Tube (7) contains saline only.

0.4 c.c. of the bacterial suspension is added to each

tube and the pipette is then sterilised. The dilutions of serum are now :

①	②	③	④	⑤	⑥	⑦
1 in 30	1 in 60	1 in 120	1 in 240	1 in 480	1 in 960	CONTROL- SUSPENSION - NO SERUM.

The mixtures are transferred with a capillary pipette to agglutination tubes, starting with tube ⑦. Incubate at 37° C. for one and a half hours and then leave for half-an-hour at room temperature, when observations are made. Some observers prefer to incubate the tubes in a water-bath at 55° C. for two hours. Clumping or agglutination can easily be detected with the naked eye; the tubes should be examined in a strong light. The flocculi also tend to sediment, and the deposit is quite perceptible in the narrow tubes.

Readings should also be made after four and twenty-four hours to detect agglutination of the O type which develops slowly (*vide* p. 34).

If less than 0.1 c.c. of serum is available, the test quantity of diluted serum and bacillary emulsion must be adjusted accordingly.

At the present time in the routine diagnosis of enteric fever, B. paratyphosus A and B are also tested simultaneously with the same series of serum dilutions as B. typhosus. The test described above is accordingly triplicated.

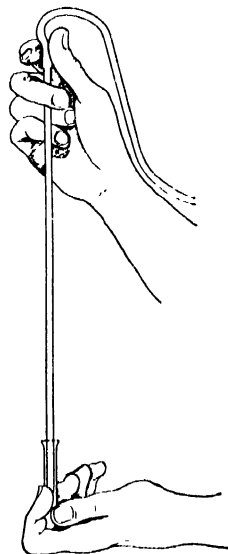
The strains of *B. typhosus*, *B. paratyphosus A* and *B* used must be standard strains of known suitability for agglutination tests.

Further references to the Widal reaction are made on pp. 200, 201, 335.

METHOD OF PIPETTING WITH A GRADUATED PIPETTE AND MOUTH-PIECE

The glass mouth-piece is held between the teeth at the right corner of the mouth, and the top of the

pipette is supported between the second and third fingers of the right hand so that the rubber tube immediately above the end of the pipette can be compressed between the thumb and the first finger (see diagram). The fluid is drawn up (*e.g.* from a test-tube) into the pipette by suction until the column extends just above the required graduation mark. The end of the mouth-piece is then closed by the tongue, and the column of fluid is depressed to the particular level by gentle pressure on the rubber tubing between the thumb and forefinger. *With the tongue still firmly applied to the mouth-piece* this exact volume of fluid can be transferred from the original tube and then expelled from the pipette into another tube.



This method, for which the necessary skill is soon acquired by practice, permits of *accurate* and *rapid* measurements of even small volumes.

The glass tube of the mouth-piece can be sterilised by flaming.

Measurement of Serum and other Fluids by drops.—Some serological workers prefer to make measurements of serum, etc., in terms of drops delivered from a suitable dropping pipette. This consists in its simplest form of a piece of quill tube drawn out to capillary dimensions (as in the capillary pipette, *vide* p. 129). It is actuated by a teat. The pipette when used is held vertically and the fluid in it is allowed to drop slowly from the capillary stem. For full details of the preparation and use of these pipettes see *A System of Bacteriology*, Medical Research Council, London, 1931, vol. ix., chap. 14.

OTHER AGGLUTINATION TESTS

The agglutination technique described above is also applicable to diagnostic tests with *B. paratyphosus* A and B, *B. melitensis*, *B. abortus*, *B. proteus* (Weil-Felix reaction of typhus fever), etc. The series of dilutions tested can, of course, be varied according to the lowest dilution in which agglutination would be accepted as a diagnostic criterion (*vide* pp. 336, 356, 373). It is essential in all cases to make these tests quantitative so that the highest dilution ("end-titre") in which agglutination occurs can be estimated.

AGGLUTINATION TESTS USED FOR THE SEROLOGICAL IDENTIFICATION OF CERTAIN ORGANISMS BY MEANS OF SPECIFIC ANTISERA

These tests are carried out by a similar technique. In this case the series of dilutions depends on the "end-titre" of the serum for the homologous organism. Thus, if the end-titre were 1 in 16,000, the following range of dilutions might be tested:—1 in 1000 to 1 in 32,000 in geometrical series. For identification the unknown organism should agglutinate in approximately as high serum-dilution as a known homologous organism.

Special applications of the agglutination technique—*e.g.* in the identification of serological types—are referred to in later chapters.

DREYER'S STANDARD AGGLUTINATION METHOD

It is shown in Chapter XIV. how certain organisms—*e.g.* *B. typhosus*—are susceptible to agglutination by normal serum, which has to be allowed for in interpreting results of diagnostic agglutination tests. Owing to the fact also that cultures of a particular organism may vary in their agglutinability, a method has been advocated by Dreyer for standardising these tests. Suspensions of the particular organisms used are prepared by growing them in bulk in a veal-extract bouillon for twenty-four hours at 37° C., and then adding 0.1 per cent. of formalin; the formalised culture is kept at 2° C., and shaken at intervals. After a few days, sterilisation is complete, and the suspension remains suitable for agglutination tests over long periods. A newly prepared suspension is compared with a standard culture as regards its agglutinability by a particular antiserum,

and any difference noted is stated as an "agglutinability factor" by which the end-titres of sera tested with the new suspension can be corrected so as to render the results comparable with those given by the standard suspension. Thus, in the actual diagnostic test, the end-titre is converted to a "reduced-titre" (R.T.) by dividing the dilution-denominator by this factor. For example, if the end-titre were 1 in 125 and the factor for the suspension 2.5, the R.T. would be 50. The agglutinability factor given to the original standard suspension is such that a reduced-titre of more than 10 would denote abnormal agglutinating power. This is arrived at after a number of tests with normal human serum. The suspensions can be obtained from the Standards Laboratory, Pathology Department, Oxford University. The agglutinability factor is stated on each bottle of suspension.

In carrying out diagnostic tests, the following series of dilutions is recommended: 1 in 25, 1 in 50, 1 in 125, 1 in 250 -- together with a control. The tubes are placed in a water-bath at 50°-55° C. for two hours and then allowed to stand at room temperature for a quarter of an hour.

These methods are fully described in the *Medical Research Council's Special Report Series*, No. 51. See also *A System of Bacteriology*, Medical Research Council, London, 1931, vol. ix., chap. 14, pp. 186-189.

STANDARD BACTERIAL SUSPENSIONS FOR TESTING "H" AND "O" AGGLUTININS¹

A drawback to the use of formalised suspensions is the fact that they may show only the "large-flake" agglutination characteristic of the H antigen (*vide* p. 34). Formalin interferes with O agglutination, and if the agglutinin formed in a case of enteric fever is mainly of the O type a formalised suspension might fail to detect it.

The H antigen can be annulled by alcohol, and if cultures are extracted with alcohol a standard suspension representing the O antigen alone can be obtained. For this purpose, growths on agar are made up and kept in the form of a dense suspension in 50 per cent. alcohol and diluted to the required degree with saline when used in the agglutination test. Formalised and alcoholised suspensions supply therefore the necessary reagents for testing H and O agglutinins.

It is advisable to use for such tests selected strains which are sensitive to H and O agglutination respectively.¹ Standard H and O suspensions can be obtained from the Standards Laboratory, Oxford University.

¹ See Felix, *Lancet*, 1930, vol. i., p. 505.

PREPARATION OF AGGLUTINATING ANTISERA

The instructions given here apply particularly to organisms of the typhoid-paratyphoid group.

Rabbits are used for immunisation, and large healthy animals should be selected, not under 2000 grams in weight.

The purity and identity of the culture used should be carefully ascertained beforehand, and in view of variability in antigenic composition the culture selected should be such that it represents the "smooth" form and the specific phase of the particular species (*vide* pp. 34, 343).

The rabbits are injected intravenously (*vide* p. 186) at intervals of five to seven days with a suspension in saline of a twenty-four-hours' slope culture killed by exposure for one hour at 60° C. The following series of doses may be given:—1/20, 1/10, 1/5, 1/3 and 1/2 culture. These doses are easily measured by emulsifying a slope culture in a given volume of saline and then injecting the appropriate fraction.

In the case of organisms of high toxicity—e.g. *B. dysenteriae* (Shiga)—it is necessary to start with even lower doses—e.g. 1/100 of a culture.

In the case of certain other organisms—e.g. *B. aertrycke*—higher titres may be obtained if living organisms are injected. To commence with, very small amounts—e.g. 0.01 c.c.—of a young living broth culture should be injected intravenously. As the animal becomes immune larger doses may be given, until several c.c. of the living culture can be tolerated.

Other methods for standardising doses may be used—e.g. where the doses are stated in terms of the number of organisms, as in the administration of vaccines (*vide* p. 228)—but the system indicated above is simple and sufficiently accurate for ordinary purposes.

Seven to ten days after the last injection a specimen of blood is withdrawn from an ear vein (*vide* p. 187), and the serum is tested for its agglutinating power towards the strain used for immunisation. A series

of dilutions is tested, and if agglutination occurs in a 1 in 1600 or higher dilution,¹ the animal is bled from the neck vessels or by cardiac puncture (*vide* p. 107), the blood is allowed to coagulate in a sterile stoppered measuring cylinder, placed overnight in the ice-chest, and the serum is then separated. 0.1 c.c. of a 5 per cent. solution of phenol in salt solution is added for each c.c. of the serum—equivalent to 0.5 per cent. pure phenol. This prevents bacterial growth resulting from any accidental contamination. The serum is stored in sterile quill tubes, about 1 c.c. in capacity, drawn out at both ends to capillary dimensions. The tubes are filled by suction, applying the mouth-piece used in pipetting (*vide* p. 198), and the ends are sealed in the Bunsen flame. Alternatively 1 c.c. or 5 c.c. stoppered bottles may be used. The serum should be kept in the refrigerator, and may be expected to retain its potency for long periods (a year or more). 50 per cent. glycerol is also used as a preservative of serum.

If the serum is dried by the methods used for complement (*vide* p. 208), its potency will be retained over considerably longer periods than if the serum is stored in the fluid state.

AGGLUTININ-ABSORPTION TESTS

Agglutinins, like other antibodies, combine firmly with their homologous antigens, and by treating an agglutinating antiserum with the homologous bacteria and then separating the organisms by centrifuging, it is found that the agglutinin has been "absorbed" or removed by the organisms from the serum.

In certain cases, to prove the serological identity of an unknown strain with a particular species, it may be necessary to show not only that it is agglutinated by a specific antiserum to approximately its end-titre but also that it can absorb from the serum the agglutinins for the known organism. This becomes necessary owing to the fact that, on immunising an animal with a particular bacterium, "group antibodies" for allied organisms are developed, and in some cases these may

¹ More powerful agglutinating sera may of course be obtained; in immunising animals, 1 in 1600 is merely the *minimum* end-titre which should be aimed at.

act in relatively high titre. "Absorption" with a heterologous strain would only remove the group agglutinins without affecting the specific agglutinin. These effects are exemplified in the *Salmonella* group (*vide* p. 342). The general method of carrying out such absorption tests is to mix a dense suspension of the organism—*e.g.* twenty-four hours' growth on a 4 in. plate of nutrient agar, suspended in 1 c.c. saline and killed at 60° C. (thirty minutes)—with an equal volume of a suitable dilution of the serum—*e.g.* sixty-four times the concentration of the known end-titre. Thus, if the end-titre is 1 in 1600, the dilution used would be 1 in 25. The mixture is incubated for three to four hours at 37° C. and the serum is then separated from the bacteria in a high-speed centrifuge. The dilution of the serum would now be approximately double the original dilution—in the example taken (*vide supra*) 1 in 50. From the treated serum a series of doubling dilutions is prepared as in direct agglutination tests (*vide* p. 197), so that, when an equal volume of bacterial suspension is added, the series will reach to the known end-titre of the serum. In the example taken above, the following series of dilutions would be prepared :

1 in 50 1 in 100 1 in 200 1 in 400 1 in 800,

and after the addition of bacterial suspension these would become

1 in 100 1 in 200 1 in 400 1 in 800 1 in 1600.

A control tube is also included, containing suspension but no serum, and the general technique is that employed in direct agglutination tests.

The serum is treated with the unknown strain, and then tested in this way with a suspension of the known organism. If the unknown strain is identical with the known type, no agglutination should occur, the specific agglutinin having been absorbed. At the same time, as a control, the serum is also treated with the known strain, and tested in the same way to ensure that absorption takes place under the conditions of the experiment. As a further control, a direct agglutination test may be made with the *untreated* serum and the homologous organism, in a parallel series of dilutions.

For an actual illustration of the application of absorption tests see p. 340.

WASSERMANN SYPHILIS REACTION

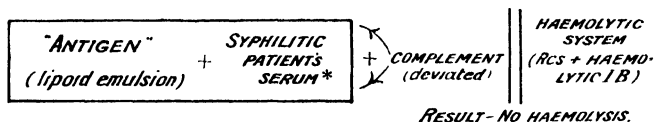
This reaction depends on the "fixation" of complement by an emulsion of certain lipoid substances (lecithin and allied substances) along with the *heated*

serum of a person infected with syphilis, and constitutes an important diagnostic test.

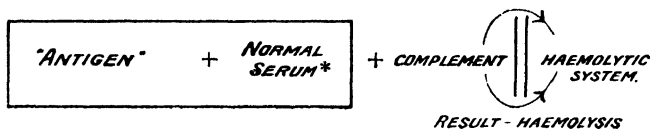
The effect is not a true immunity reaction, though the lipid emulsion *plus* syphilitic serum fixes complement in the same way as a bacterial or other antigen *plus* its homologous antiserum (*vide* p. 35). Possibly the lipid plays the part of a hapten in this reaction. Though some observers attribute the phenomenon to a physico-chemical change in the serum the evidence suggests that it depends on the presence in the syphilitic serum of a "lipoidophile" antibody-like principle which is stable at 55° C.

For complement-fixation tests, an indicator of the presence of complement is required. The "haemolytic system" used in these tests serves this purpose. It consists of the red corpuscles of a particular animal species "sensitised" with the corresponding haemolytic immune body (I.B.)—*e.g.* the red cells of the ox *plus* the serum of a rabbit which has been immunised with ox red cells. The immune body in the serum is thermostable. The serum is heated at 55° C. to annul the natural complement, and stored in tubes (*vide infra*). The heated serum is non-haemolytic by itself, but in the presence of a suitable complement produces lysis of the homologous red corpuscles. Fixation of complement is denoted by the absence of lysis in the haemolytic system.

In its simplest form the Wassermann reaction can be represented as follows:—



* Previously heated at 55° C.



It should be specially noted that the Wassermann reaction is both qualitative and quantitative. Not only does the test indicate whether the reaction is positive or not, but the various degrees of strength of the reaction may also be determined, from a strong positive (+ + +) to a weak positive (+) or a doubtful positive ($\pm$). This quantitative testing is most important in assessing the value of treatment, or the completeness of cure.

The technical application of the reaction demands a very accurate standardisation of each reagent. Further, the amount of complement used in relation to the quantities of antigen and serum must be adjusted with such delicacy that the weakest reactions can be accepted as specific.

Although the essential principles and reagents are the same, several modifications of the technique are employed, which are concerned mainly with the volume of the reagents and the method of measurement of the quantities used.

The majority of workers use constant amounts of antigen and of patient's serum, with varying amounts of complement. In the standard method recommended by the Royal Medico-Psychological Association for mental hospital practice,¹ constant amounts of antigen and of complement with varying amounts of patient's serum are employed.

The different forms of technique may be classed as follows :

- (1) Where the quantities are measured by exact delivery of pipetted amounts.
- (2) Where the reagents are all diluted to a standard volume, variation in actual amount—*e.g.* of complement—being effected by altering the concentration in the standard volume.

Thus, only one standard volume is required instead of varying quantities, and moreover

¹ See S. A. Mann and F. Partner, *Memorandum on the Wassermann Reaction in Mental Hospital Practice*, London County Council, 1931 (obtainable from King & Son, London).

the method can be applied to small amounts, but it has the disadvantage that it can become grossly inaccurate in the hands of other than skilled workers.

The preparation of the reagents is essentially the same in the different methods.

Two widely used examples of the different systems of measurement are given in detail.

METHOD I. (*reagents measured in exact pipetted amounts*)

ANTIGEN

(1) 20 grams of *sheep heart-muscle*, carefully freed from fat and fibrous tissue, are *finely* ground with sand in a mortar, and extracted for four days at room temperature with 100 c.c. of 96 per cent. alcohol. In this way lecithin and similar substances are extracted from the tissue. The extract is filtered, and pure cholesterol is dissolved in it to the point of saturation. The cholesterol acts by intensifying the "antigenic" properties of the tissue extract.

For the test, a suspension is prepared by adding 1 part of the alcoholic extract to 12 parts of normal saline solution. In preparing the emulsion the *maximum turbidity* should be obtained by running the extract slowly on to the salt solution in a cylindrical measure (or test-tube), and then mixing slowly by rotation of the cylinder held in a slanting position.

(2) The following alternative antigen is also recommended: *human heart-muscle* from the left ventricle (cause of death immaterial) is freed from visible fat, minced finely and ground for a minute in a mortar with absolute alcohol (1 gram of heart to 9 c.c. of alcohol) and clean sand. The mixture is shaken in a shaking machine for $1\frac{1}{2}$ hours and then filtered. To 1.5 volumes of this extract is added 1 volume of 1 per cent. cholesterol in absolute alcohol. For use,

1 volume of this mixture is placed in a beaker and 29 volumes of saline are measured into a cylinder, the contents of which are then poured rapidly into the beaker. In the actual test, as shown on p. 212, this antigen suspension can be used in place of that described above.

PATIENT'S SERUM

A specimen of blood is obtained usually by vein puncture (*vide* p. 141) as for blood culture. The blood is then placed in a sterile stoppered test-tube and allowed to coagulate. It is advisable to obtain about 5 c.c. of blood. The serum is pipetted off after separation and heated in a water-bath at 55° C. for half-an-hour. Heating *eliminates the fallacy of non-specific fixation effects which may occur with normal unheated sera plus the antigen*; it also deprives the serum of its own complementing property.

COMPLEMENT

Fresh guinea-pig serum is used. It contains an active haemolytic complement for the red corpuscles of the ox or sheep sensitised with the homologous haemolytic antibody. The blood is obtained, twelve to eighteen hours before the test, by severing the large vessels of the neck over a 6 in. funnel, from which the blood is collected in a measuring cylinder; it is allowed to coagulate and stand overnight in the ice-chest. The complement in serum too recently withdrawn is apt to be excessively "fixable," and in consequence is unsuitable for the Wassermann test.

It should be noted that complement is unstable and deteriorates on keeping at ordinary temperatures. It is advisable throughout the experiment to keep the guinea-pig serum on ice.

Preservation of Complement.—Complement can be preserved by keeping the serum frozen at -15° to -20° C. A more convenient way of preserving complement is to dry it by Craigie's

or Hartley's methods. The essential principle of these methods is to dry the guinea-pig serum *in vacuo* over fused calcium chloride or phosphorus pentoxide. The vacuum (obtained by means of a Geryck pump) should be within 1-2 mm. of mercury from perfect. The water vapour arising from the serum is immediately absorbed by the calcium chloride or phosphorus pentoxide, so that the rapid vaporisation causes the serum to freeze solid within ten to twenty minutes. The water vapour still continues to arise from the solid material, so that in a few hours the serum is completely dry. The secret of success is the rapid freezing of the complement and the subsequent drying from the frozen state.

In Craigie's method, the guinea-pig serum having separated overnight in the refrigerator is pipetted into a previously cooled thin Petri dish (without a cover). The serum should not be more than 3 mm. deep. The dish is supported on three corks, which act as heat insulators, in a large desiccator in which freshly-fused calcium chloride has been placed. The lid is now put on, and the bulk of the air extracted by means of an ordinary water-pump. The desiccator is connected to the Geryck pump and the vacuum maintained by continuous pumping for about twenty minutes. Within this time the serum will have frozen solid. The tap of the desiccator is then turned off, the Geryck pump disconnected and the desiccator placed in the refrigerator. Next day the complement will be completely dry. The dry powder can conveniently be stored in a stoppered tube or small screw-capped bottle in a closed jar of fused calcium chloride. To reconstitute the serum add 1 part of dried complement to 15 parts of distilled water.

Hartley's method is essentially the same, except that to the guinea-pig serum are added sodium chloride crystals 3.4 per cent. The salted serum is pipetted very accurately in amounts of 1 c.c. into round-bottom wide-necked ampoules of about 7 c.c. capacity. The ampoules are then arranged in a thin glass dish, around which is packed 2 parts ice and 1 part salt, so that the complement is frozen solid. The dish containing the ampoules is then placed in a desiccator over phosphorus pentoxide, and the exhausting and drying carried out as above, the desiccator being left in the refrigerator for about ten days. When the drying process is complete the ampoules are sealed. To reconstitute the serum the ampoules are opened, and 5 c.c. of distilled water added, which produces a 1 in 5 dilution of complement in normal saline. By this method no weighing out of the dried product is necessary, and preparations have been obtained which have retained their activity for over a year.

HAEMOLYTIC SYSTEM

With guinea-pig complement a haemolytic system consisting of ox or sheep red corpuscles, sensitised with the appropriate immune serum is used.

Defibrinated blood is obtained at the abattoir (*vide* p. 102) and washed three or four times with normal saline, being centrifuged after each washing to separate the corpuscles. The centrifuged deposit of corpuscles after the final washing is suspended in normal saline to form a 3 per cent. suspension, and five minimum haemolytic doses (M.H.D.) of the immune serum are added.

Burroughs Wellcome haemolytic serum for *sheep* red corpuscles may conveniently be used in preparing a haemolytic system for the test. Anti-sheep haemolytic sera tend to exert a pronounced agglutinating effect on the homologous corpuscles with rapid sedimentation of the cells; we prefer, therefore, to use for complement-fixation tests ox corpuscles with an anti-ox haemolysin obtained from the rabbit.

The method of preparing a HAEMOLYTIC ANTISERUM and of estimating its M.H.D. may be summarised as follows:—a rabbit is injected intravenously at weekly intervals with increasing amounts of washed red cells—*e.g.* 0.5 c.c., 1.0 c.c., 1.5 c.c. of the sediment after washing and centrifugalisation. A week after the last injection, a small quantity of blood is withdrawn from an ear vein; the serum is separated, and its M.H.D. estimated by testing the haemolytic effect of varying amounts (*e.g.* from 0.001 c.c. to 0.005 c.c.) on 1 c.c. of a 3 per cent. suspension of red cells along with an excess of guinea-pig complement (*e.g.* 0.05–0.075 c.c.) (*vide* methods given below). As guinea-pig serum contains natural haemolysins for ox and sheep corpuscles, the complement-containing serum must previously be freed from this antibody by mixing equal volumes of the guinea-pig serum and the appropriate red corpuscles (washed), keeping the mixture at 0° C. for one hour and then separating the serum by centrifuging. If the M.H.D. is 0.001 c.c.—0.002 c.c. the animal is bled from the neck vessels or by cardiac puncture (*vide* p. 107). The blood serum is separated, heated for one hour at 55° C., and stored in sealed quill tubes (*vide* p. 203).

THE TEST

Apparatus required:

Small test-tubes, $3 \times \frac{1}{2}$ in.; a rack for the tubes; 1 c.c. pipette graduated to the tip in 1/10ths and 1/100ths; 0.1 c.c. pipette graduated in 1/100ths and 1/500ths (*vide* p. 196).

As specimens of complement from different guinea-pigs vary quantitatively in their haemolytic effect, it is necessary first to estimate the M.H.D. of the complement to be used. The M.H.D. is the smallest amount required to produce complete lysis of the given quantity of red cells in the presence of excess of immune body. This usually lies between 0.004 c.c. and 0.01 c.c. of undiluted guinea-pig serum for 0.5 c.c. of the haemolytic system.

To measure amounts less than 0.01 c.c. make a 1 in 10 dilution of the serum, and of the diluted serum take ten times the actual amount required, using the 0.1 c.c. pipette graduated in 1/100ths and 1/500ths.

The following exemplifies the determination of the M.H.D. of complement:---

Tube	1	2	3	4	5
Haemolytic system	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Complement (diluted 1 in 10)	0.02 c.c.	0.04 c.c.	0.06 c.c.	0.08 c.c.	0.1 c.c.
Haemolysis	Trace	Marked	Just complete	Complete	Complete

The M.H.D. is therefore 0.06 c.c. of a 1 in 10 dilution of complement —i.e. 0.006 c.c. undiluted complement.

Tubes 1 and 5 could be omitted from the series unless the complement is unusually active or weak.

The actual Wassermann test is shown in the Table below.

Full Test

Tube . .	Test proper				Serum Control		Antigen Control	
	1	2	3	4	1	2	1	2
Normal saline .	—	—	—	—	0.5 c.c.	0.5 c.c.	—	—
Antigen suspension	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.	—	—	0.5 c.c.	0.5 c.c.
Patient's serum .	0.05 c.c.	0.05 c.c.	0.05 c.c.	0.05 c.c.	0.05 c.c.	0.05 c.c.	—	—
Complement	2 MHD	4 MHD	8 MHD	14 MHD	2 MHD	4 MHD	2 MHD	4 MHD

Complement dose Control :—

Tube	1	2	3	4
Saline	0.5 c.c.	0.5 c.c.	0.5 c.c.	0.5 c.c.
Complement (diluted 1 in 10). .	0.04 c.c.	0.06 c.c.	0.08 c.c.	0.1 c.c.

Abridged Test

Where large numbers of Wassermann tests have to be carried out, and there is need for economy in labour and materials, the test may be abridged as follows : tube 4 of the "test" series is omitted and 6 M.H.D. of complement are substituted for 8 M.H.D. in tube 3 ; tube 2 of the "serum control" may also be omitted, though this may sometimes introduce a difficulty if a serum is anticomplementary ; tube 4 of the complement dose control could be omitted unless the complement is unusually weak.

The mixtures are incubated at 37°C. for one and a half hours, and then to each tube 0.5 c.c. of the haemolytic system is added. The tubes are again incubated for one hour, when the results are noted. Absence of haemolysis in the test series signifies that

**EXAMPLES OF THE RESULTS OF WASSERMANN TESTS
CARRIED OUT BY THE METHOD DESCRIBED**

<i>Tube</i>	<i>Test proper</i>				<i>Serum Control</i>		<i>Antigen Control</i>	
	1	2	3	4	1	2	1	2
Amount of complement in M.H.D. . .	2	4	8	14	2	4	2	4
Amount of undiluted complement in c.c.	0.012	0.024	0.048	0.084	0.012	0.024	0.012	0.024
Negative serum	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis
Positive serum .	No lysis	No lysis	Partial lysis	Complete lysis	Complete lysis	Complete lysis	-	-
"Weak positive" serum . . .	No lysis	Partial lysis	Complete lysis	Complete lysis	Complete lysis	Complete lysis	-	-
"Marked positive" serum .	No lysis	No lysis	No lysis	No lysis	Complete lysis	Complete lysis	-	-

Complement dose :—

Undiluted complement
in c.c.

0.004
Marked
lysis

0.006
Just complete
lysis

0.008
Complete
lysis

In this experiment the M.H.D. of undiluted complement is 0.006 c.c. both in the initial titration and in the complement-dose control.

fixation of complement has occurred, so that not even 1 M.H.D. is left free. The serum and antigen controls determine any possible anti-complementary action of either of these reagents, and the complement dose control indicates any possible deterioration in the haemolytic value of the complement by dilution and incubation. By carrying out the test in this quantitative way the number of doses of complement fixed by antigen *plus* serum can be estimated—i.e. the strength of the reaction. Known negative and positive sera must always be included in each set of Wassermann tests as controls.

The practical details and interpretation of the results can be learned only by actual acquaintance with the test.

It must be remembered that specimens of complement from different guinea-pigs vary in fixability. With a weakly fixable complement it may even be impossible to elicit positive reactions, while with a strongly fixable complement non-specific effects are rendered more marked. The controls with known negative and positive sera are therefore indispensable.

Any anti-complementary effect of the serum or antigen must be allowed for in estimating the fixation by serum *plus* antigen, and the results with known positive and negative sera are to be regarded as standards for comparison with the results given by the sera tested.

The criteria of positive and negative reactions suggested by Browning and Kennaway may be followed: a serum which permits of complete lysis with the same minimum amount of complement as the negative control serum is, of course, negative; a serum which requires at least twice as much complement as the negative control serum to yield complete lysis is to be regarded as positive. Sera which show intermediate effects may be classified as "doubtful." If the serum in question exhibits any degree of anti-complementary effect, allowance must be made for this.

THE WASSERMANN REACTION WITH CEREBRO-SPINAL FLUID

In testing spinal fluid the reaction is carried out as in the case of serum, but with the following modifications :—

- (1) The antigen is prepared by emulsifying 1 part of the cholesterolised sheep heart extract with 12 parts of spinal fluid ; 0.5 c.c. of this mixture replaces antigen + serum in the usual test.

The alternative antigen (2) is prepared by mixing 1 part of the cholesterolised extract with 29 parts of the fluid.

- (2) In place of saline + serum in the usual serum controls, 0.5 c.c. of spinal fluid is tested.
- (3) The cerebro-spinal fluid is not heated to 55° C. before the test as in the case of serum.

METHOD II. (*standard volume method*)

HARRISON'S TECHNIQUE MODIFIED BY WYLER ¹

It is not possible to include all the minutiae of detail described by Wyler, and workers who use his method should consult his monograph. The main essentials of reagents and technique however are given below.

In this test a standard volume of each reagent is used, and 0.11 c.c. is the most convenient amount. An ordinary capillary pipette is graduated with mercury and may be used with the rubber tube and mouthpiece as described on p. 198, or with a rubber teat. Instead of adding different amounts of the same dilution of complement, as in the method previously described, the same amount of different dilutions is taken. For example, suppose the M.I.D. were the standard volume of a 1 in 60 dilution of complement, then 3 M.I.D. would be present in the standard volume of a 1 in 20 dilution, while for 5 M.H.D. a 1 in 12 dilution would be used.

¹ For full details of this method see "The Wassermann Test," by E. J. Wyler, *Medical Research Council's Special Report Series*, No. 129, 1929 ; also *Reports on Public Health*, No. 67, 1932.

Antigen—

An alcoholic extract of *human heart-muscle* is prepared similar to that described on p. 207 (2). For use, place in a wide tube 0·3 c.c. antigen and 0·2 c.c. 1 per cent. solution of cholesterol in absolute alcohol. Add very rapidly 7 c.c. of saline. This 1 in 15 dilution is the antigen suspension referred to in the description of the test proper.

Patient's serum—

This is inactivated and a 1 in 5 dilution in saline is used.

Complement—

Fresh guinea-pig serum (p. 208).

Haemolytic system—

Sheep red corpuscles, 3 per cent., are sensitised with 6 M.I.D. immune body. The cell suspension is standardised by means of a Haldane haemoglobinometer. Either Burroughs Wellcome haemolytic serum, or rabbit *v.* sheep antiserum, can be used.

Titration of complement—

Prepare 2 c.c. of a 1 in 10 dilution of the guinea-pig serum in saline. Set out eight tubes. Add to each respectively, starting from the left, 1 volume of each of the following dilutions of complement prepared from the 1 in 10 dilution: 1 in 30, 1 in 40, 1 in 50, 1 in 60, 1 in 70, 1 in 80, 1 in 90, 1 in 100. To each tube add 2 volumes of saline, and 1 volume of sensitised red cells. Prepare a similar series of tubes with the complement dilutions, but instead of adding 2 volumes of saline add 1 volume of antigen suspension, and 1 volume of saline. Then add 1 volume of sensitised red cells and shake thoroughly. Incubate both sets of tubes in the water-bath for half-an-hour at 37° C. and read the result. In the first set of tubes (*i.e.* without antigen) the highest dilution showing complete haemolysis is the minimum haemolytic dose. Dilutions of complement equivalent to 2, 3 and 5 M.H.D. are now prepared from the undiluted guinea-pig serum. The second set of tubes (with antigen) indicates whether the antigen suspension has any anticomplementary action.

Test proper—

Four tubes are required for each test, two for serum controls, and two for the antigen-serum mixture. The contents of each tube are as follows:

- | | |
|--------------------------|----------------------------------|
| Tube 1 (serum control) . | 1 volume saline. |
| | 1 volume patient's serum diluted |
| | 1 in 5 in saline. |
| | 1 volume complement diluted to |
| | 3 M.H.D. |
| Tube 2 (serum control) . | Same as tube 1, but the comple- |
| | ment present is 2 M.H.D. |

Tube 3.	1 volume patient's serum diluted 1 in 5. 1 volume complement 5 M.H.D. 1 volume antigen suspension.
Tube 4.	Same as tube 3, except that complement is 3 M.H.D.

The reagents are added in the following order :

- (1) 1 volume of dilution of patient's serum into all tubes.
- (2) 1 volume of saline into tubes 1 and 2.
- (3) 1 volume complement dilution 2 M.H.D. into tube 2.
- (4) 1 volume complement dilution 3 M.H.D. into tubes 1 and 4.
- (5) 1 volume complement dilution 5 M.H.D. into tube 3.
- (6) 1 volume antigen suspension into tubes 3 and 4.

The racks are shaken after each set of ingredients is added to the tube.

The tubes are incubated first at room temperature for thirty minutes and then in the water-bath at 37° C. for thirty minutes, after which 1 volume of sensitised red cells is added, and the tubes well shaken and replaced in the water-bath at 37° C. After one or two minutes they are taken out, quickly shaken, and replaced. The results are read as soon as the No. 1 tubes and some of the No. 4 tubes, including the No. 4 tube of the negative control, show complete haemolysis—usually in about five minutes.

In addition to the test proper, described above, in which known positive and negative sera are used as controls, an antigen control and a corpuscle control are also carried out at the same time.

Antigen control—

To one tube are added 1 volume saline, 1 volume 2 M.H.D. complement and 1 volume antigen. This is placed in the rack with the test proper, 1 volume of red cells being added later along with the rest of the tubes.

On incubation there should be complete haemolysis within thirty minutes, confirming that the antigen does not absorb more than one dose of complement.

Corpuscle control—

This consists of 1 volume of sensitised red cells with 3 volumes of saline. The red cells are added to the saline at the same time as to the other tubes. The tube is centrifugalised when the tests have been read. The supernatant fluid must be colourless, showing that no deterioration has occurred since the complement was titrated.

METHOD OF RECORDING THE RESULTS OF THE TEST

Symbols—

- + + = Complete inhibition of lysis with 3 M.H.D. complement, and complete or almost complete inhibition with 5 M.H.D. complement.
 + = Complete or almost complete inhibition of lysis with 3 M.H.D. complement and partial inhibition of lysis with 5 M.H.D. complement.
 ± = Partial inhibition of lysis with 3 M.H.D. complement; slight inhibition or complete lysis with 5 M.H.D. complement.
 - = Complete lysis with 3 M.H.D. complement.

Interpretation of signs—

- + + = Strong positive
 + = Positive
 ± = Positive in *known* cases of syphilis only. In unknown cases it is regarded as *doubtful*, and as an indication for further investigation.
 - = Negative, but the patient is not necessarily non-syphilitic.

Test with cerebro-spinal fluid—

The fluid does not require to be heated.

Into a series of tubes place varying quantities of the cerebro-spinal fluid as follows :

- 2 volumes undiluted fluid.
- 1 volume undiluted fluid.
- 1 volume 1 in 2.5 dilution.
- 1 volume 1 in 5 dilution.

To each tube are added 1 volume antigen and 3 M.H.D. complement. As a control another tube with 2 volumes of undiluted fluid and 3 M.H.D. complement, but without antigen, is used. The remainder of the procedure corresponds to the serum test, and the strength of the reaction is judged by the number of tubes, excluding the control, in which a positive reaction occurs. A strongly reacting fluid gives a positive result with the 1 in 5 dilution, while a weakly reacting specimen yields fixation of complement only in the tube containing 2 volumes of undiluted fluid.

Partial or even complete inhibition of lysis in the tube containing 2 volumes of undiluted cerebro-spinal fluid cannot be accepted as positive for diagnostic purposes unless there is also definite inhibition of lysis in the tube containing 1 volume of undiluted fluid.

For the technique of carrying out the test, using dropping pipettes, Wyler's monograph (see footnote, p. 215) should be consulted.

THE FLOCCULATION TEST FOR SYPHILIS

Since the discovery of Michaelis (1907) that precipitation or flocculation occurs when a syphilitic serum is treated with organ extracts, various tests depending on this phenomenon have been described and recommended for the routine serum diagnosis of syphilis, as substitutes for the Wassermann reaction—*e.g.* the Sachs-Georgi reaction, the Sigma reaction of Dreyer and Ward,¹ the Kahn reaction,² the Meinicke test, etc. Most of these have been reviewed in the League of Nations report on the sero-diagnosis of syphilis.³ The differences in technique depend mainly on the manner in which the organ extract is prepared, and the method by which the resulting flocculation, precipitation, or clarification (Meinicke test) is observed. The method of the Bacteriology Department, Edinburgh University, may be recommended as a simple and reliable technique. The results are practically parallel to those of the complement-fixation test, and the flocculation reaction may be substituted for it, provided the serum can be tested within two days after withdrawal of the blood specimen. In treated cases of syphilis the flocculation reaction may be definitely positive when the Wassermann reaction is very weak or negative; sometimes, however, the reverse result is obtained. It is to be noted that the flocculating property of syphilitic serum is rapidly lost if specimens of blood are kept for several days.

The "antigen" is similar to that used for the Wassermann reaction.

20 grams of sheep's heart-muscle are ground with sand in a mortar and extracted for four days at room temperature with 100 c.c. of 96 per cent. alcohol. The extract is filtered and 0.25 gram cholesterol is added and dissolved by keeping the extract

¹ *Lancet*, 1921, vol. 2, p. 956.

² Kahn, *The Kahn Test*, Baltimore, 1928.

³ *League of Nations Publications*, 1928, No. C.II.726; and 1931, No. C.II.968.

in a water-bath at 55° C. for half-an-hour. It is then allowed to stand at room temperature for a day and again filtered.

The optimum dilution of this antigen for the test is usually 1 in 18, but may vary for different preparations, and must therefore be ascertained for each new preparation. Likewise the "sensitiveness" must be compared with a known standard so that, if necessary, the antigen can be corrected or standardised.

Standardisation.—Equal parts of the cholesterolised extract and normal saline are rapidly mixed together and the mixture is allowed to stand for ten minutes. 0·2 c.c. of this mixture is added to each of five tubes containing (1) 1 c.c., (2) 1·3 c.c., (3) 1·6 c.c. (4) 1·9 c.c., (5) 2·2 c.c. of saline respectively, the series representing final dilutions of 1 in 12, 1 in 15, 1 in 18, 1 in 21 and 1 in 24. These are shaken, and their opacities compared with a suspension of a standard antigen made up similarly in its optimum dilution (e.g. 1 in 18). The particular dilution in the series with which the standard suspension matches is the optimum of the new antigen preparation. In the test one part of antigen suspension is added to two parts of serum, so that the antigen suspension actually used is three times the concentration of the estimated optimum—e.g. if the optimum is 1 in 18, a 1 in 6 dilution would be used.

The sensitiveness depends on the content of extracted lipid and on the cholesterol dissolved by the alcohol of the extract. A lipid content of 0·82 to 0·87 per cent., a cholesterol content of 0·2 to 0·25 per cent. and a total lipid-cholesterol content not exceeding 1·07 per cent. yield a preparation of standard sensitiveness. A new preparation is compared with a standard by testing it in parallel with several known positive sera. If the results are not quantitatively similar, 20 c.c. of the extract both before and after cholesterolisation are evaporated to dryness in weighed dishes so that the content of lipid and lipid-cholesterol can be determined.

By the addition of (1) a concentrated extract containing 1·5 per cent. lipid, or (2) uncholesterolised extract or (3) 1 per cent. cholesterol, the composition of the new preparation can be so adjusted that the content in lipid and cholesterol is the optimum stated above. The dilution required for the test is again determined and the sensitiveness is also compared with the standard antigen. For further details of the method of standardising this antigen reference should be made to the *Indian Journal of Medical Research*, vol. 17, p. 477: "The Standardisation of Antigen used in the Syphilis Flocculation Reaction," by K. V. Krishnan.

The reaction is carried out in a manner similar to an agglutination test (*vide* p. 197).

The activity of the serum is at an optimum when heated at 54°–56° C. For the test the patient's serum is therefore heated at 55° C. for half-an-hour, and a series of concentrations is prepared in small test-tubes ($3 \times \frac{1}{2}$ in.) as follows (physiological saline solution being used as the diluent):—

①	②	③	④	⑤	⑥
1 in 2	1 in 4	1 in 8	1 in 16	1 in 32	1 in 64

0.4 c.c. of each concentration is a convenient quantity for the test, and to each tube one-half of this volume (0.2 c.c.) of the antigen suspension is added. If the optimum dilution of the antigen is 1 in 18, then a 1 in 6 suspension is used; it is prepared by mixing rapidly equal quantities of the undiluted antigen and saline solution, allowing the mixture to stand for ten minutes, and then adding sufficient saline to give the required dilution (*i.e.* 1 in 6). The mixtures of serum and antigen are transferred to narrow agglutination tubes, incubated at 37° C. for four hours, and allowed to stand overnight at room temperature, when readings are made of the results. A final reading should be made after thirty-six hours. In reading the results, the tubes should be held in front of a shaded lamp so that they are brightly illuminated without the glare of the lamp reaching the eyes. A control tube containing antigen suspension and saline but no serum should be included. With strongly reacting sera, flocculation may occur even in tube ⑥. With weak sera definite flocculation may occur only in tube ①. "Zone phenomena" are sometimes noticed, especially with sera after three days or more from the time of withdrawal of the blood—*i.e.* flocculation is less with lower dilutions of serum than with higher dilutions.

The reaction can be hastened by shaking the mixtures of serum and antigen for five to ten minutes before incubation. This can easily be done after the addition

of the antigen to the serum dilutions, by placing the small test-tubes vertically in a shaking machine (such as that used for shaking a Winchester quart bottle). The tubes are held in racks fixed to the carrier of the machine.

The test can, if necessary, be abridged by omitting tubes 5 and 6.

THE MEINICKE CLARIFICATION TEST FOR SYPHILIS

The Meinicke clarification reaction is a development of the Meinicke turbidity reaction. The organ extract is made from ox heart-muscle; balsam of tolu is added to it, and the system is adjusted with alkali of varying strength until the isoelectric point of the colloidal suspension is approached, with the result that a positive reaction presents a clear-cut precipitation of the colloidal elements and clarification of the system.

The test can be set up in two forms:

A. The macro. reaction, a procedure which requires 0·7 c.c. of serum and takes sixteen to twenty hours to obtain a complete and quantitative reading.

B. The micro. reaction, which should be utilised only where the amount of serum available is insufficient for the macro. test, as in pediatric practice, or where a rapid test is required. The reaction enables a result to be obtained within one hour.

The micro. reaction should not be carried out as a routine procedure, but only in exceptional cases, and will not be described here.

It is claimed that haemolytic, icteric and turbid sera are all suitable for the reaction, and further that a falsely positive result cannot be produced by the admixture of haemoglobin with the sera. Nevertheless, the sera should be thoroughly centrifuged and used as fresh as possible. In general, there is fair agreement between the Wassermann and Meinicke results, but in treated cases this concordance does not occur; a positive Meinicke reaction may persist in a case in which the Wassermann reaction has become negative following treatment. The Meinicke clarification test, however, can be commended as a parallel test to the Wassermann reaction on account of its simplicity of manipulation, clarity of results and general reliability.

DETAILS OF TECHNIQUE

(1) *Reagents required.*—(a) The antigen is specially prepared and standardised at Dr Meinicke's Serological Institute, and can be obtained from Messrs Bredt, 38 Great Tower Street, London, E.C.3. It should be kept at room temperature and in the dark, preferably in the 20° C. incubator, in which the tests should be made.

(b) The serum must not be heated or inactivated in any way, but must be quite fresh.

(c) Sodium chloride solution. Meinicke suggests keeping a stock solution of 10 per cent. NaCl, and diluting to 3.5 per cent. when needed. A stock solution of the strength desired for the test (3.5 per cent.) obviates the possibility of error in preparation, and keeps just as well as the stronger solution. Sodium chloride (analytical reagent) is used and is dissolved in distilled water. It is convenient to put up a batch in 500 c.c. screw-stoppered bottles, which can be kept for some months. For identification, a dark-blue bead and a red bead (p. 80) are added.

(d) Sodium carbonate solution. A stock solution of 1 per cent. anhydrous sodium carbonate in distilled water is prepared, and will keep indefinitely in a stoppered bottle. It should be stored in the dark and in the cold.

The solutions for use in the test are 0.01 per cent., 0.015 per cent., 0.02 per cent. and 0.04 per cent. sodium carbonate in 3.5 per cent. NaCl, and should be freshly prepared each week, as follows:

Take four 50 c.c. clean dry cylindrical measures and label them with a grease pencil respectively 10, 15, 20 and 40. Into the first pipette 0.5 c.c., into the second 0.75 c.c., into the third 1.0 c.c., and into the fourth 2.0 c.c. of the 1 per cent. sodium-carbonate solution. Fill each cylinder to the 50 c.c., mark with 3.5 per cent. sodium-chloride solution, and mix thoroughly.

(2) *The Macro. Test.*—Each serum to be tested requires four tubes. Into the first three tubes pipette 0.2 c.c. of the serum, and into the fourth 0.1 c.c. serum and 0.1 c.c. of the 3.5 per cent. NaCl solution, to which has been added 0.04 per cent. sodium carbonate. When all the sera to be tested have been pipetted into the necessary tubes, the dilutions of antigen can be prepared in batches. This is done by placing into four wide tubes 10 c.c. of each of the four sodium-carbonate-saline solutions and into other four tubes 1 c.c. of the heart extract reagent. These are placed in the water-bath at 56° C. and heated until they reach this temperature. Each tube of saline-carbonate solution is rapidly mixed with 1 c.c. antigen and immediately put back into the 56° C. water-bath for exactly two minutes. They are placed in a rack on the bench and are then ready to be used in the test. These details of preparation are important, and must be closely followed. The antigen suspensions thus prepared are:

A.	A saline antigen suspension containing	0.010	} per cent. sodium carbonate
B.	" " " "	0.015	
C.	" " " "	0.020	
D.	" " " "	0.040	

For each test pipette 0.5 c.c. of suspension A into No. 1 tube,

0.5 c.c. of *B* into No. 2 tube, 0.5 c.c. of *C* into No. 3 tube, and 0.5 c.c. of *D* into No. 4 tube. This pipetting must be performed very rapidly, and all the antigen dilutions should be in their respective tubes in not more than ten minutes from the time of preparation, hence the necessity for making "batches" of the antigen of not more than 11 c.c.—this will complete twenty-two tests. After the reagent has been added the tubes are shaken and placed in an incubator at 20° C. for twenty hours. If the tests are left at room temperature care should be exercised that it does not fall below 18° C.

(3) *Controls*.—Pipette into four separate tubes 0.5 c.c. of each of the antigen dilutions. After twenty hours at 20° C. the tube containing 0.01 per cent. sodium carbonate should show almost complete flocculation with sedimentation, and clearing of the supernatant fluid; the tube with 0.015 per cent. sodium carbonate should show slight clearing with a relative amount of sedimentation, and the other two tubes should retain their opacity. This is a control of the sensitivity of the reagent. For the actual test the controls consist of known positive and negative sera. With the negative sera the opacity remains in all tubes, whereas with positive sera a clearly discernible clarification occurs in one or more of the tubes.

(4) *Reading the Results*.—The test-tubes having been left standing for twenty hours at 20° C. the reading of the reaction is performed with the naked eye, looking at the tubes with a window behind them.

Negative reactions. The fluid in the tubes is opaque as before; there may be a slight sedimentation and a slightly lighter shade of the fluid. The decisive fact is that notwithstanding the sedimentation the fluid never becomes transparent in a negative case.

Positive reactions. A complete clarification of the suspension represents a positive reaction, and may occur in all or any of the four tubes.

(5) *Interpretation of Results*.—If no clarification of the fluid has occurred in any one of the four test-tubes containing the serum under examination, the reaction must, of course, be recorded as *negative*.

Complete clarification of all four tubes, or of the fourth tube only, should be interpreted as *strongly positive* (+ + + +).

The distinctive sign of medium and weak reactions is that the clarification of the fluid is most distinct in the first tube with 0.01 per cent. of sodium carbonate, and that by degrees it decreases, with increasing sodium carbonate concentration.

Complete clarification in the first three tubes must be interpreted as (+ + +). A result of (+ +) is recorded when the first two tubes are completely clear, and (+) when the first tube alone shows clarification.

(6) *Procedure with Small Amounts of Serum.*—Should the amount of serum available be less than 0·7 c.c. the test can be performed by using the first, second and fourth tubes, or even the second and fourth only, this requiring only 0·3 c.c. serum.

THE COMPLEMENT-FIXATION TEST IN TUBERCULOSIS

This reaction has been utilised in the diagnosis of certain cases of tuberculosis, and also as an index of the activity of the disease.

The principle of the test is that governing complement-fixation reactions generally, and a method corresponding to that used in the Wassermann reaction is employed. The *antigen* consists of tubercle bacilli and their products. Various antigen preparations have been advocated. We would recommend that of Besredka, prepared by growing tubercle bacilli in egg broth.¹

Serum is obtained as for the Wassermann reaction (*q.v.*) and heated at 55° C. for half-an-hour.

Complement.—Guinea-pig serum is used, as in the Wassermann test.

Haemolytic System.—Exactly as in the Wassermann test. The following table shows the scheme of the test and controls :

	<i>Test</i>				<i>Serum Controls</i>		<i>Antigen Controls</i>	
Tube . .	1	2	3	4	1	2	1	2
Saline .	—	—	—	—	0·3 c.c.	0·3 c.c.	—	—
Antigen .	0·3 c.c.	0·3 c.c.	0·3 c.c.	0·3 c.c.	—	—	0·3 c.c.	0·3 c.c.
Patient's serum (55° C.)	0·1 c.c.	0·1 c.c.	0·1 c.c.	0·1 c.c.	0·1 c.c.	0·1 c.c.	—	—
Complement .	2 MHD	3 MHD	4 MHD	6 MHD	2 MHD	3 MHD	2 MHD	3 MHD

Note.—A tube with 8 MHD of complement may be added to the test series.

¹ *Compt. Rend. Acad. d. Sci.*, 1913, vol. 166, p. 1633.

This antigen is obtainable from La Biothérapie, 3 Rue Maublanc, Paris, through Sealand Trading Ltd., 24 Holborn, London, E.C.1.

A complement-dose control is also included, as in the Wassermann test.

After incubation for one and a half hours, 0.5 c.c. haemolytic system is added, and the tubes are again incubated for one hour.

Known negative and positive control sera should be included in each set of tests as standards for comparison.

The following examples show the types of results met with :

Tube . . .	Test				Serum Controls		Antigen Controls	
	1	2	3	4	1	2	1	2
Doses of complement .	2	3	4	6	2	3	2	3
Negative .	Complete	.	.	.	Complete	.	Complete	.
Negative .	Partial	Complete	.	.	Partial	Complete	Almost complete	.
Weakly positive .	Trace	Partial	Complete	.	Complete	.	Complete	.
Positive . .	Nil	Trace	Partial	Complete	Complete	.	Complete	.
Markedly positive .	Nil	Nil	Nil	Partial	Complete	.	Complete	.

Note.—"Complete," "partial," "trace" and "nil" refer to resulting lysis.

Antigens prepared from the tubercle bacillus may yield positive reactions with strongly-positive Wassermann sera ; a Wassermann test, therefore, should also be carried out.

THE COMPLEMENT-FIXATION TEST IN GONORRHOEA

This test is analogous to the complement-fixation reaction in tuberculosis. The antigen is prepared from cultures of the

gonococcus and the test is carried out by the same methods as those described above for other complement-fixation reactions, the actual procedure varying according to the choice of technique made by the worker. Further reference to this test is made on p. 280.

PREPARATION OF BACTERIAL VACCINES

The method to be described refers mainly to the preparation of vaccines on a small scale, such as autogenous vaccines—*i.e.* consisting of the organism or organisms isolated from a particular patient and used for the treatment of the case (*vide* p. 27).

The organism must be isolated in pure culture, and then several cultures are made on appropriate solid medium so as to yield sufficient growth after twenty-four to forty-eight hours' incubation, according to the amount of vaccine to be prepared, and the abundance of the growth on the particular medium. The growth is emulsified in sterile saline solution (0.85 per cent. sodium chloride) so as to form a fairly dense emulsion. The emulsion should be free from fragments of medium; if present they can be removed by centrifugalising the emulsion for two or three minutes, or by allowing them to sediment by gravity, and then decanting the supernatant fluid. The bacterial suspension must be rendered as uniform as possible by shaking in a tube or bottle with glass beads. A special shaking machine is generally used for this purpose. *All manipulations involved in preparing the suspension must be carried out with strict precautions to avoid contamination.*

STANDARDISATION

It is necessary at this stage to estimate the *approximate* number of bacteria per c.c. of the suspension. Various methods are available for this purpose. Those recommended are:

1. *Haemocytometer Method.*—Use a Thoma cell or the central portion of any of the rulings on the Bürker pattern of haemocytometer; make a 1 in 20 dilution

of the suspension—0·1 c.c. suspension + 1·8 c.c. saline + 0·1 c.c. centrifugalised Löffler's methylene blue, adding two loopfuls of undiluted formalin to kill the organisms; mount a drop on the haemocytometer stage as in blood-counting and enumerate the organisms in 20-50 small squares in different parts of the cell; determine the average number per square and multiply this by 80,000,000; the result is the approximate number of bacteria per c.c. in the original suspension.

2. *Comparison with Standard Opacity Tubes (Brown).*¹—This consists in comparing the opacity of the suspension with that of a series of ten standard tubes containing different dilutions of suspended barium sulphate. In making comparisons the bacterial suspension should of course be placed in a tube

SHOWING THE RELATION OF OPACITY TO THE NUMERICAL EQUIVALENT OF VARIOUS BACTERIA ESTIMATED BY MEANS OF THE HAEMOCYTOMETER METHOD

Opacity Tube No.	<i>Streptococcus aureus</i>	<i>Streptococcus haemolyticus</i>	<i>Pneumococcus</i>	<i>Gonococcus</i>	<i>E. coli</i>	<i>E. typhosus</i>	<i>E. paratyphosus B</i>	<i>Sh. catarrhalis</i> (on ord. agar)	<i>B. typhosus</i> (100,000)
10	3789	3043	7053	3578	3787	4577	4171	3611	11,396
9	3410	2739	6348	3220	3408	4119	3754	3250	10,256
8	3031	2434	5642	2862	3030	3662	3337	2889	9117
7	2652	2130	4937	2505	2651	3204	2920	2528	7977
6	2273	1826	4232	2147	2272	2746	2503	2167	6838
5	1895	1522	3527	1789	1894	2289	2086	1806	5698
4	1516	1217	2821	1431	1515	1831	1668	1444	4558
3	1137	913	2116	1073	1136	1373	1251	1083	3419
2	758	609	1411	716	757	915	834	722	2279
1	379	304	705	358	379	458	417	361	1140

The figures represent millions per c.c.

Standard opacity tubes with the corresponding tables are supplied by Burroughs Wellcome & Co.

¹ See H. C. Brown, *Indian Journal of Medical Research*, 1919, vol. 7, pp. 238-250.

similar to the standards. The matching is facilitated by reading printed letters through the suspensions.

The Table opposite gives the numerical equivalents of the opacity standards for certain organisms according to Cunningham and Timothy.¹

STERILISATION OF THE BACTERIAL SUSPENSION

The suspension is sterilised at relatively low temperatures—*e.g.* 60° C. for one hour in a water-bath. To ascertain whether the organisms have been killed, six loopfuls are transferred to a tube of suitable medium and incubated for forty-eight hours. (For further sterility tests *vide infra*.)

PREPARATION OF THE VACCINE FOR ADMINISTRATION

Any series of graduated doses consisting of a certain number of organisms (usually computed in millions) can be prepared in volumes of 1 c.c. by making appropriate dilutions in carbol-saline (0.85 per cent. sodium chloride + 0.5 per cent. phenol) from the original standardised suspension. Graduated pipettes, as used in serological work, are employed for the purpose. The dilutions are made in sterile tubes and each dose is transferred to a sterile vaccine ampoule which is then sealed.

The most convenient method of supplying the vaccine for actual use is to prepare from the stock suspension, concentrations of 50, 100, 500 or 1000 million organisms per c.c. (according to the doses required) in quantities of 20 c.c. The dilutions are placed in 25 c.c. "vaccine bottles" with special tightly-fitting thick rubber caps which are covered with a layer of paraffin wax. The required dose can be obtained by puncturing the cap with the hypodermic syringe and withdrawing the appropriate amount.

When a vaccine representing more than one type of organism is required—*e.g.* for mixed infections—pure cultures of each organism must be obtained and

¹ See *Indian Journal of Medical Research*, 1924, vol. 11, p. 1253.

separate standardised suspensions prepared. Appropriate concentrations of each are then combined in the final preparation.

In preparing dilutions from the stock vaccine all manipulations, etc., must be carried out with strict precautions to prevent contamination. Pipettes, tubes, ampoules, bottles, caps, etc., must be absolutely sterile.

Before supplying the diluted vaccine it is essential to carry out further sterility tests, using the contents of one of the ampoules, or 2 c.c. withdrawn from the bottled vaccine with a syringe. One-half of this sample is tested for aerobic organisms and one-half for anaerobes by appropriate cultural methods.

The regulations under the Therapeutic Substances Act 1925 applicable to the "manufacture for sale" of various therapeutic substances, including vaccines (see Second Schedule Therapeutic Substances Regulations, 1927), lay down *standard sterility tests* which should be followed:

"The tests shall be made on fluid media, the quantity of medium contained in each tube or other vessel used in the test being such as to secure that any phenolic anti-septic present in the sample is diluted to less than 0.01 per cent.

In the case of a test for aerobic organisms the medium shall consist either of a meat extract with the addition of 1 per cent. of peptone, or of such an equivalent as can be prepared by the tryptic digestion of muscle. After the final sterilisation the hydrogen-ion concentration of the medium shall be between the limits represented by $pH=7.2$ and $pH=7.8$.

In the case of a test for anaerobic organisms the medium shall consist of a nutrient broth similar to that used in testing for aerobic organisms, with the addition of heat coagulated muscle of an amount sufficient to occupy a depth of not less than 1 centimetre at the bottom of the tube. After the final sterilisation the hydrogen-ion concentration of the medium shall be between the limits represented by $pH=7.2$ and $pH=7.8$.

Before the test inoculation the medium shall be heated to $100^{\circ}C$. for a period sufficient to free it completely from dissolved oxygen, and then cooled to $37^{\circ}C$. or lower.

The inoculated tubes shall be incubated at $37^{\circ}C$. for seven days. . . ."

CHAPTER VIII

BACTERIOLOGICAL EXAMINATION OF WATER AND MILK; TESTING OF ANTISEPTICS

BACTERIOLOGICAL EXAMINATION OF WATER

FROM the hygienic standpoint, the bacteriological examination of water resolves itself into the determination of the presence or absence of any serious excretal contamination.

Under certain conditions, *B. coli* represents the most reliable indicator of such pollution.

As this group of organisms is derived from the intestine of various animals, even water supplies far removed from human contamination may contain *B. coli* in small numbers. In water grossly polluted with excretal matter (e.g. sewage), *B. coli* is present in large numbers. If it is to be accepted as an indicator of impurity, the test for its presence must be carried out on a quantitative basis, so that the *B. coli* content of the water can be definitely estimated.

This group of bacteria includes a large number of different types. Some of these ("typical") are more prevalent in faeces than others ("atypical") and obviously the former are of more significance as indicators of recent faecal contamination. In carrying out the test for *B. coli* in water, it is essential to determine whether the types present are "typical" or "atypical" (*vide* pp. 323, 325).

The presence of streptococci and sporing anaerobic bacilli—e.g. *B. welchii*—is of additional significance in determining water purity. The occurrence of streptococci is of course proof positive of recent faecal

pollution, but their absence does not exclude such impurity. The intestinal sporing anaerobes, being highly resistant, do not by themselves necessarily indicate a recent or serious pollution.

The enumeration of the total viable bacteria in a water specimen is also a useful supplementary test in determining its purity, but this test is of little value by itself.

The demonstration of pathogenic bacteria (e.g. *B. typhosus*) would obviously constitute the most direct proof of a serious impurity, but pathogens, if present, are usually so scanty that the technical difficulty of their isolation makes this test impracticable for ordinary purposes.

The routine tests used in the bacteriological examination of water are:

- (1) Enumeration of the viable bacteria.
- (2) The quantitative *B. coli* test.

Collection of specimens.—Specimens are taken in 6 oz. stoppered bottles sterilised by dry heat, the stopper and neck of the bottle being covered by two layers of kraft paper (*vide* p. 74). When water is drawn from a tap, the mouth of the tap should be flamed and the water allowed to run for ten minutes before filling the bottle. In the case of streams, rivers and lakes, the bottle is filled by dipping the mouth (with the stopper in position) below the surface, and then removing the stopper under water with forceps. This avoids the collection of surface water, which may contain a good deal of decomposing vegetable matter. When a sample is to be obtained from a depth, a bottle weighted with lead is used, having two cords attached—one to the neck, the other to the stopper; the bottle is lowered to the required depth, and is filled by jerking out the stopper by means of the attached cord; the bottle is then quickly raised to the surface and re-stoppered.

When a certain length of time (over three hours) must elapse before the laboratory examination can be carried out, the bottles should be kept on ice. Special

ice-boxes for the purpose can be obtained, and are essential where specimens have to be transported some distance.

Enumeration of viable bacteria.—With a sterile pipette add 1 c.c. water to 10 c.c. nutrient agar (standardised to a pH 6.6–7.0), melted and cooled to 45° C.; mix thoroughly, pour in a Petri dish, and allow to solidify.

If the water is suspected of contamination, add a smaller quantity—*e.g.* 0.1 c.c.—and in dealing with specimens of uncertain purity it is advisable to make a series of plate cultures with varying quantities of the water. The agar should be as transparent as possible.

Make duplicate plates in this way and incubate one at 37° C. for two days, the other at 20° C., for three days. Nutrient gelatin in place of agar is often used for determining the number of organisms viable at 20° C.

Count the colonies that develop in the medium. To aid counting, divide the plate into sections by ruling on the glass with a grease pencil. Count the colonies in each section (using a hand lens if necessary to detect small colonies) and add the numbers. Each colony may be taken to represent one viable bacterium in the original specimen.

The number of viable bacteria in the average municipal water supply—*e.g.* after filtration—should not usually exceed 100 per c.c. A count of 1000 per c.c. or more may arouse suspicion regarding the purity of a water supply, but on the whole the results of such enumeration are of relatively little value by themselves in estimating the hygienic purity of a water.

In uncontaminated water, since the organisms present are mainly saprophytes, the number of colonies developing at 20° C. is usually much greater than at 37° C., the ratio being higher than 10:1. A contaminated water may yield a ratio less than this—*e.g.* 10:3.

B. Coli* Test—**Media required:***

- (1) 2 per cent. peptone water containing 0·5 per cent. sodium taurocholate, 1 per cent. lactose, and neutral red or Andrade's solution as indicator (*vide* p. 96).
- (2) Same constituents as (1), but in double concentration.

Fixed amounts of these media are put up in 50 c.c. sterile cylindrical bottles stoppered with cotton-wool plugs, and containing a small inverted tube for indicating gas production. The amounts are as follows:—

5 c.c. of the single strength medium		
5 c.c. of the double strength medium		
10 c.c.	”	”
25 c.c.	”	”

The medium, after bottling, is sterilised at 100° C. for twenty minutes in the Koch steriliser on three successive days. With sterile pipettes the following amounts of water are added¹:—

0·1 c.c. to	5 c.c. of the single strength medium		
0·5 c.c. ”	5 c.c.	”	”
1 c.c. ”	5 c.c.	”	”
5 c.c. ”	5 c.c. of the double strength medium		
10 c.c. ”	10 c.c.	”	”
25 c.c. ”	25 c.c.	”	”
25 c.c. ”	25 c.c.	”	”

If it is desired to prove the absence of *B. coli* from a larger amount (100 c.c.)—*e.g.* in a filtered water—two additional bottles containing 25 c.c. of the specimen may be included in the series.

The bottles are incubated for forty-eight hours, and sub-inoculations are then made on MacConkey plates

¹ This should be done if possible at the place where the water specimen is taken, especially if it is some distance from the laboratory.

from those which show acid, or acid and gas production. The plates are incubated for twenty-four hours, and red colonies are picked off on to agar slopes, and the resulting pure growths are put through the following tests to ascertain whether they represent "typical" *B. coli* :—

Fermentation of $\left\{ \begin{array}{l} \text{Lactose} \\ \text{Inosite} \end{array} \right.$
 Production of *Indole*
 Voges-Proskauer reaction
 Liquefaction of *Gelatin*
(vide pp. 125, 323)

The fermentation of lactose may be assumed from the original test, but it should be confirmed by a subsequent test with the pure culture isolated.

A typical *B. coli* shows the following reactions :—

Lactose	+	(acid and gas)
Inosite	—	
Indole	+	
Voges-Proskauer reaction	—	
Gelatin	—	

The smallest amount of water which contains typical *B. coli* can then be stated ; but it is, of course, only permissible to report that a given quantity contains *B. coli* when all the larger volumes also yield a growth of the organism.

The Application of the Methyl-Red and Voges-Proskauer Reactions

In determining the type of *B. coli*, use has been made of the methyl-red and Voges-Proskauer reactions. The methyl-red reaction indicates the hydrogen-ion concentration produced by growth in a standard glucose medium. The organism is grown for three days in a 0.5 per cent. peptone-water medium containing 0.5 per cent. glucose and 0.5 per cent. di-potassium hydrogen phosphate. Part of the resulting growth is tested for the Voges-Proskauer reaction (*vide* p. 324), and to the remainder two drops of a methyl-red solution are added (0.1 grm. methyl-red dissolved in 300 c.c. alcohol and then made up to 500 c.c. with distilled water). A red colour denoting a

high hydrogen-ion concentration is described as “positive”; yellow, signifying a low concentration, is “negative.” The prevalent type of *B. coli* in excreta is “methyl-red positive, Voges-Proskauer negative.”

Citrate-Utilisation Test for Differentiating Typical and Atypical B. Coli

This test has also been used for separating typical and atypical forms of *B. coli* in the examination of water supplies. It depends on the ability of such coliform bacilli as *B. lactis aerogenes* to utilise citrate as a source of carbon¹ and to grow in a synthetic medium containing this salt, the typical forms being unable to grow under these conditions.² The medium recommended by Koser consists of 1.5 grams sodium ammonium hydrogen phosphate (microcosmic salt), 1 gram potassium di-hydrogen phosphate, 0.2 gram magnesium sulphate and 2 grams sodium citrate in 1000 c.c. distilled water. Tubes used should be cleaned by boiling in sodium hypochlorite and then rinsed with distilled water. *B. aerogenes* types grow and produce a visible turbidity in the medium within ten days.

A pure water should not contain *B. coli* in 25 c.c. or less. Waters of high purity usually show absence of *B. coli* in 50 c.c. In the case of a specimen from a suspicious source with typical *B. coli* in 10 c.c. the water can justifiably be condemned for drinking purposes. When typical *B. coli* is found in 1 c.c. or less the water must be regarded as unsafe for drinking.

Examination for Streptococci

Varying quantities of water, as in the *B. coli* test, are added to a 1 per cent. glucose-bouillon medium which is incubated for forty-eight hours. Streptococci can be detected by the examination of hanging-drop preparations. The minimum amount of water containing streptococci may thus be stated as in the *B. coli* test.

Examination for B. welchii

50 c.c. of water are added to 100 c.c. of sterile milk in a stoppered conical flask of suitable size. The flask is then heated

¹ See H. C. Brown, 1921, *Lancet*, vol. 1, p. 22.

² See S. A. Koser, 1923, *Journ. Bacteriol.*, vol. 8, p. 493; and *Ibid.*, vol. 9, p. 59.

at 80° C. for fifteen minutes. Sterile liquid paraffin is run on to the surface of the medium to maintain anaerobiosis. On incubation the occurrence of the "stormy-clot" reaction is indicative of the presence of *B. welchii* (*vide* p. 392). To examine larger quantities of water, additional flasks of milk are inoculated each with 50 c.c. of the specimen and treated as above.

For methods of isolating the typhoid-paratyphoid bacilli and *V. cholerae* from water, see pp. 338, 363.

BACTERIOLOGICAL EXAMINATION OF SEWAGE AND SEWAGE EFFLUENTS

The bacteriological examination of sewage is not of great practical importance, unless in determining the purity of an effluent from a sewage purification process.

The procedure is the same as in water examination; an estimation of the viable bacteria present is made by plating and counting colonies, and the *B. coli* test is carried out as with a specimen of water; much smaller amounts, however, are tested than in the case of water, depending on the likely extent of dilution of the effluent. The numbers of bacteria per c.c. in crude sewage vary greatly—*e.g.* from 1 to 100 millions.

Typhoid and paratyphoid bacilli may be isolated from communal sewage by methods of selective culture. For this purpose the brilliant-green enrichment method (*vide* p. 333) may be recommended.

BACTERIOLOGICAL EXAMINATION OF MILK

In hygiene work the bacteriological examination of milk generally consists in :

- (1) An estimation of the number of viable bacteria present in a given quantity.
- (2) An estimation of the degree of contamination present from faecal sources—*e.g.* a quantitative *B. coli* test.
- (3) The determination of the presence of specific pathogenic organisms—*e.g.* *B. tuberculosis*.

The specimen of milk is collected in an 8 oz. sterile stoppered or screw-capped bottle, and, should there be any unavoidable delay in carrying out the tests after collection of the sample, the bottle must be kept on ice; if it has to be transported some distance, a special ice-box should be used similar to that employed for the transmission of water specimens.

Dilutions of 1 in 10, 1 in 100, 1 in 1000 and 1 in 10,000 (with sterile water as diluent) are prepared in sterile stoppered bottles, by adding 10 c.c. of the undiluted milk to 90 c.c. water—*i.e.* 1 in 10, 10 c.c. of the 1 in 10 dilution to 90 c.c. water—*i.e.* 1 in 100, and so on. The dilutions are thoroughly mixed by shaking.

1. The number of bacteria per c.c. capable of growing in a suitable nutrient agar medium is estimated by plating 1 c.c. of each of the decimal dilutions (as in water examination), incubating for forty-eight hours at 37° C., counting the colonies in those plates which contain from about thirty to three hundred colonies, and calculating from this the number of viable organisms per c.c. of the milk, assuming that the number of colonies represents the number of viable organisms originally present.

The medium used is as follows: peptone 0.5 per cent., Lemco 0.3 per cent. and agar 1.5 per cent., set to a reaction of pH 6.8.¹

The colony count should be carried out with the aid of a good artificial illuminant, and a hand-lens should also be used.

According to J. F. Malcolm (*Journ. Dairy Research*, 1932, vol. 4, p. 91) a more accurate estimate of the bacterial content is obtained by making five plates from the 1 in 100 dilution, and two plates from the 1 in 1000 dilution, and taking the mean count. In samples whose counts amount to 50,000 per c.c. this gives reliable

¹ A milk-agar prepared by adding 10 c.c. sterilised separated milk to each litre of the nutrient agar yields higher colony counts than the ordinary medium, and gives a better representation of the degree of bacterial contamination present (see *Ministry of Agriculture Bulletin*, No. 46, 1932).

results. When the count is about 100,000 accurate estimates require five plates also from the 1 in 1000 dilution.

Under the most favourable conditions a specimen of milk may contain at least 500 bacteria per c.c.; but under bad conditions the numbers may reach even several million per c.c. Milk that has been produced with special precautions to avoid undue contamination is recognised for consumption as "certified milk." Thus, "certified milk" must not contain more than 30,000 bacteria per c.c. when delivered to the consumer or at any time prior to delivery (*vide infra*).

2. Varying amounts of milk are added to tubes or bottles of lactose-bile-salt medium (as in the *B. coli* water test, p. 234). The range of amounts that require to be tested depends on the likely degree of contamination. In the case of ordinary dairy milk the following series is suggested:—

1.0 c.c.	} Undiluted milk		
0.1 c.c.			
0.1 c.c. of a 1 in 10		dilution of the milk	
" "	1 in 100	" "	
" "	1 in 1000	" "	
" "	1 in 10,000	" "	
" "	1 in 100,000	" "	

The decimal dilutions are prepared in series (*vide supra*).

The smallest amount which yields acid and gas production (due to *B. coli*) is ascertained.

3. *Examination for Tubercle Bacilli*.—The sample is thoroughly mixed and a quantity of 100 c.c. is divided into 50 c.c. amounts and centrifugalised for half-an-hour at a minimum speed of 3000 revolutions per minute. The sediment in each tube is suspended in 3 c.c. of sterile saline solution. (A microscopic examination of the sediment for tubercle bacilli may be made at this stage, but as shown later is quite unreliable. Before staining, the dried films should be treated with ether for some minutes to remove the fat.) Two guinea-pigs are injected subcutaneously on the inner side of one thigh with 3 c.c. of suspended

sediment and kept under observation to ascertain whether tuberculous lesions result (*vide* p. 298). One guinea-pig is killed at the end of four weeks, and an autopsy carried out; if it shows no tuberculous lesions the other animal is kept for eight weeks, when it is killed and examined.

An alternative method is to centrifuge 100 c.c. as above and suspend the deposit in 2.5 c.c. of saline. The resultant emulsion is injected intramuscularly into the thighs of two guinea-pigs. The animals are kept for five weeks, when both are killed and examined.

Lesions should be examined microscopically for tubercle bacilli to confirm their tuberculous nature. (It has been shown that *B. abortus*, which may occur in cow's milk, produces tubercle-like lesions in guinea-pigs.) It is necessary to inoculate at least two animals from one specimen, as inoculated guinea-pigs may die sometimes from infection with other organisms present in the milk—*e.g.* sporing anaerobic bacilli. This difficulty may be obviated by treating the sediment with antiformin (*vide* p. 302) before injection. Some intercurrent disease—*e.g.* pneumonia, enteritis, etc.—may also cause death before tuberculous lesions have developed and so nullify the test if only one animal is injected.

Some of the cream from the centrifugalised milk may also be used for the inoculation.

The microscopic examination may reveal acid-fast bacilli other than the tubercle bacillus (*vide* p. 305). The absence of tubercle bacilli in films does not exclude their presence in the specimen. The inoculation test, therefore, is the only valid method of demonstrating tubercle bacilli in milk.

Other pathogens in milk.—The methods for demonstrating *B. typhosus* (and *B. paratyphosus*) and *B. diphtheriae* correspond to those used for the isolation of these organisms. For *B. typhosus*, the sediment, after centrifugalisation, should be plated out on the surface of MacConkey's medium and at the same time tubes of brilliant-green peptone water are inoculated

as in isolating the organism from faeces (*vide* pp. 332, 333). For *B. diphtheriae*, plates of tellurite medium are inoculated with the sediment (*vide* p. 113).

B. abortus may be demonstrated in milk by inoculating two guinea-pigs as in the test for the tubercle bacillus (*vide supra*). The animals are killed and examined after four and eight weeks respectively, cultures are obtained from the spleen by the appropriate method (*vide* p. 374) and the organism is then identified. An agglutination test with the serum of the inoculated guinea-pig and *B. abortus* affords evidence of infection from the milk without the necessity of isolating the organism.

BACTERIOLOGICAL STANDARDS

The following standards have been laid down by the Ministry of Health for the control of the "graded" milks:—

Certified Milk.—Viable bacteria not to exceed 30,000 per c.c.; *B. coli* to be absent from 0·1 c.c.

Grade A Milk. Viable bacteria not to exceed 200,000 per c.c.; *B. coli* to be absent from 0·01 c.c.

Grade A Pasteurised.—Same standard as for Certified Milk.

Ordinary Pasteurised Milk.—Viable bacteria not to exceed 100,000 per c.c.

TESTING OF ANTISEPTICS

By "germicide" is meant a substance which destroys micro-organisms. The terms "antiseptic" "bactericide," and "disinfectant" are usually employed as synonyms, though "antiseptic" is more accurately applicable to substances which inhibit bacterial growth. Most substances, however, which inhibit growth are germicides if used in sufficiently high concentration, or if the exposure is sufficiently long.

The "INHIBITION COEFFICIENT" is the lowest percentage concentration of a particular antiseptic or germicide that will completely inhibit growth in nutrient medium—*e.g.* 0·25 per cent. carbolic acid for *B. typhosus*.

One of the best methods of determining this is to incorporate a series of different concentrations of the antiseptic in 10 c.c.

nutrient agar, pour the medium in plates, and then make a stroke inoculation from a bacterial emulsion. The emulsion should be prepared in sterile distilled water from a young culture on solid medium, and should show just a faint turbidity to the naked eye. Different organisms can be tested at the same time by making stroke inoculations from each on the various plates. The surface of the medium must be free from condensation fluid.

The plates are incubated for forty-eight hours, when observations can be made.

Instead of solid medium, tubes of bouillon (carefully standardised as regards pH) or serum (sterile ox serum previously heated at 56° C. for several hours) may be substituted. To a series of such tubes varying concentrations of the antiseptic are added and then each is inoculated with a standard amount of bacterial suspension. The effect is observed after incubation at 37° C. for forty-eight hours, the presence or absence of living organisms being decided by subculturing; one stroke is made from a loopful of each mixture. In this way one agar plate will serve for subcultures from a series of tubes.

In the case of slowly acting bactericidal substances, such as acriflavine and other antiseptic dyes, it is often found that concentrations which have entirely inhibited growth, as shown by their complete transparency, still contain a few living organisms as tested by subculture. The lowest concentration of antiseptic which produces this result (inhibitory concentration) yields a satisfactory index of the potency.

The "INFERIOR LETHAL COEFFICIENT" expresses the concentration of an antiseptic and the time of exposure required to kill non-sporing bacteria—*e.g.* 1 : 60 carbolic acid usually kills haemolytic streptococci in five minutes.

It can be determined by preparing a series of concentrations of the substance in a fixed quantity of diluent (*e.g.* sterile water), adding a constant quantity of bacterial emulsion, and transferring at intervals a certain amount of the mixture to some suitable culture medium (*e.g.* bouillon; or melted agar at a temperature of 45°C., which, after the transfer, is poured in plates).

The quantity of antiseptic carried over to the culture medium must be less than the inhibition coefficient.

The "SUPERIOR LETHAL COEFFICIENT" expresses the concentration of a germicide and the time of exposure required to kill bacterial spores—*e.g.* 1 : 2000 perchloride

of mercury has been found to kill *B. anthracis* spores in twenty-six hours.

The "PHENOL COEFFICIENT" expresses the germicidal power of a particular substance as compared with pure phenol.

The method of estimating this coefficient is known as the RIDEAL-WALKER test.¹

It should be used only for testing disinfectants composed of coal-tar derivatives.

Materials Required —

- (1) Standard bouillon, made as follows : —

Lab. Lemco	20 grams
Peptone (Euppton) ²	20 grams
Sodium chloride	10 grams
Distilled water	1000 c.c.

Boil for thirty minutes, cool, and make up to 1 litre with freshly boiled distilled water. Make neutral at 37 °C. to phenol phthalein with normal sodium hydroxide solution. Steam for thirty minutes to precipitate phosphates, and filter whilst hot. Cool, and adjust pH to 7·6 with normal hydrochloric acid. Distribute in 5 c.c. amounts into tubes and sterilise in the autoclave or steamer.

- (2) Standard platinum loop of 28 S.W.G. wire, 4 mm. internal diameter, bent almost at a right angle to the wire, so that in the subsequent manipulations the plane of the loop is horizontal.
- (3) Phenol. Pure phenol having a crystallising point of 40·5° C. must be used.
- (4) Culture of *B. typhosus*. It is of the utmost importance to employ a standard culture, and the "Lister" strain of *B. typhosus*³ must be used.

Subcultures should be made in the standard bouillon, at twenty-four-hours' intervals, three times before the test is carried out, and a twenty-four-hours' broth culture used for the test proper.

¹ For full details of technique, see *British Standards Specification*, No. 541, "Determination of the Rideal-Walker Coefficient," 1934; obtainable from British Standards Institution, 28 Victoria Street, London, S.W.1.

² Allen & Hanburys.

³ Obtained from the Curator, National Collection of Type Cultures, Lister Institute, Chelsea Bridge Road, London, S.W.1.

Method of Testing—

- (1) Determine beforehand the inhibition coefficient of the particular germicide for the standard strain of *B. typhosus* and make up a series of five graded concentrations, the lowest being slightly greater than the inhibition coefficient (*vide infra*).

The strongest concentration in the series is made up in a sterile stoppered flask with sterile distilled water as the solvent or diluent, and from this the remaining concentrations are prepared by appropriate dilution with sterile water in sterile flasks or tubes. The necessary measurements are made with sterile graduated pipettes.

- (2) Make up 100 c.c. of a 5 per cent. stock solution of the pure phenol in sterile distilled water, and from it prepare the following dilutions of phenol :

1 in 95, 1 in 100, 1 in 105, 1 in 110, and 1 in 115.

These dilutions must not be used if more than a week old.

- (3) To 5 c.c. (in stoppered sterile test-tubes) of each of the solutions prepared from the germicide to be tested, add with a sterile pipette 0.2 c.c. of the twenty-four hours' broth culture of *B. typhosus* and shake the mixtures. The tubes containing the solutions should be kept during the test in a water-bath at 18° C.
- (4) At intervals of 2½ minutes up to 10 minutes remove a large loopful from each mixture, using the standard platinum-wire loop of 4 mm. diameter, and transfer to tubes of 5 c.c. standard bouillon.
- (5) and (6) Carry out with the phenol solutions the same procedure as in (3) and (4).

The bacterial emulsion in (3) should be added to the tubes of antiseptic in succession at definite intervals—*e.g.* 30 seconds. The loop-transfers to bouillon from each tube, after 2½, 5, 7½ and 10 minutes respectively, can then be accurately timed.

- (7) Incubate the bouillon tubes for forty-eight hours, and note those in which growth has occurred.
- (8) The coefficient is calculated as follows :

Divide the figure indicating the degree of dilution of the disinfectant which shows life in 2½ and 5 minutes but no life thereafter *by* that figure indicating the degree of dilution of phenol which shows life in 2½ and 5 minutes but no life thereafter.

The following results illustrate the test :—

<i>Dilution</i>			<i>Time in Minutes</i>			
			2½	5	7½	10
Unknown Germicide	1:400	. .	-	-	-	-
	1:500	. .	-	-	-	-
	1:600	. .	+	-	-	-
	1:700	. .	+	+	-	-
	1:800	. .	+	+	+	+
Phenol	1:95	. .	+	-	-	-
	1:100	. .	+	+	-	-
	1:105	. .	+	+	+	-
	1:110	. .	+	+	+	-
	1:115	. .	+	+	+	+

(+ = growth, - = no growth)

$$\text{Phenol coefficient} = \frac{700}{100} = 7.0.$$

PART III

THE PATHOGENIC AND COMMENSAL MICRO-ORGANISMS (INCLUDING THE FILTERABLE VIRUSES) AND BACTERIO- LOGICAL DIAGNOSIS

CHAPTER IX

THE PYOGENIC COCCI AND OTHER ORGANISMS ASSOCIATED WITH THE COMMONER SUPPURATIVE CONDITIONS; ORGANISMS ASSOCIATED WITH CONJUNCTIVITIS; PNEUMOCOCCUS

PYOGENIC COCCI

THESE organisms are found in various acute inflammatory and suppurative conditions. They are associated with pus formation, and are therefore designated "pyogenic." The most frequent are :

Staphylococcus (pyogenes) aureus, and
Streptococcus haemolyticus.

STAPHYLOCOCCUS AUREUS

Morphology.—Spherical cocci arranged in irregular clusters, the individual cells being approximately 0.8–0.9 μ in diameter. Single forms and pairs may also be noted.

Staining.—Gram-positive.

Culture.—Aerobic and facultative anaerobic; temperature range—10°–42° C., optimum—37° C.; growth occurs on ordinary nutrient media.

Agar stroke—thick, opaque, moist, shiny, "oil-paint"-like growth which develops a characteristic golden or orange-yellow colour.

Colonies on agar—circular discs, relatively large even after 24 hours (2–3 mm. in diameter), and presenting the same characters as the stroke culture.

Colonies on blood-agar—similar to those on agar but somewhat larger; definite orange-yellow colour; marked zone of haemolysis (*vide infra*).

Gelatin—growth in this medium is associated with liquefaction due to a proteolytic ferment (gelatinase).

On potato medium, growth is similar to that on agar; on this medium pigmentation is specially marked.

Bouillon—uniform turbidity, with subsequent orange-coloured deposit.

Milk—acid formation, and coagulation.

Coagulated serum is liquefied.

Haemolysin is produced in culture.

Various carbohydrates are fermented (with acid but without gas production)—*e.g.* glucose, lactose, saccharose, mannite.

Viability.—The thermal death-point is about 62° C., but some strains are more resistant to heat—*e.g.* withstanding 75° C. for a short time. Laboratory cultures survive for months. The organism is fairly resistant to drying. It is killed within a few minutes by 5 per cent. phenol.

Other Types of Staphylococci

Staphylococcus (pyogenes) albus.—Growth is similar to that of *Staphylococcus aureus*, but white in colour. This type is generally less active than the *aureus* strains in the following properties: liquefaction of gelatin, fermentation of sugars, haemolysin production; on blood-agar, may or may not show a narrow zone of haemolysis.

Strains of staphylococci corresponding to *S. albus* and occurring as commensals on the skin have been named *S. epidermidis*.

Staphylococcus citreus.—An uncommon type; differentiated from others by lemon-yellow colour of growth; does not generally liquefy gelatin.

Staphylococcus cereus albus and *Staphylococcus cereus flavus* (*Micrococcus flavus*).—Uncommon types; develop wax-like growths, the former white, the latter yellow. They do not liquefy gelatin.

Toxins of Staphylococci

Certain strains of the *aureus*, *albus* and *citreus* types are markedly toxigenic. Culture-filtrates produce haemolysis, killing of leucocytes, necrosis of tissue—e.g. when injected into the skin—and a rapidly lethal effect on intravenous injection. The question is unsettled whether these effects are due to different toxic principles or a single exotoxin.

A special medium (Walburn's) has been found most suitable for the preparation of staphylococcal toxin; this consists of a meat extract prepared from ox heart to which are added 0.5 per cent. Witte's peptone, 0.2 per cent. potassium dihydrogen phosphate and 0.03 per cent. magnesium sulphate; the pH is adjusted to 6.8. The culture is grown in an atmosphere of 10–20 per cent. carbon dioxide.

Cases of food-poisoning have been reported which have been due to the toxins of staphylococci growing in the incriminated article of food, and pronounced local and general toxic effects have been observed in experimental animals after the introduction of staphylococcal culture-filtrates into the stomach.

Occurrence of Staphylococci

In localised abscesses, wound suppuration, skin pustules, furuncles and carbuncles, blepharitis, mucous catarrhs, acute osteomyelitis and periostitis, septicaemia, pyaemia, urinary sepsis, etc. *Staph. aureus* is the type usually found in pyogenic lesions.

The staphylococci generally are common skin, mouth and throat commensals, especially the *albus* type, and are found in air and dust and on clothing, and on eating and drinking utensils. The *aureus* type is the most virulent; the others are either non-pathogenic or of relatively low virulence.

STAPHYLOCOCCUS ASCOFORMANS

This organism is associated with the condition of "botryomycosis" prevalent in equines. In the tissues the cocci are frequently capsulate and, especially in chronic lesions, occur in zoöglœa-like masses or clusters. These aggregates may resemble in naked-eye appearance actinomyces "granules" (*vide* p. 404). In culture, capsules are not seen and the organism resembles *S. aureus* in its general characters.

A similar staphylococcal infection, pathologically resembling actinomycosis, may occur in the udder of the cow.

STREPTOCOCCUS HAEMOLYTICUS

Morphology.—Spherical or oval cocci, $0.7-1\mu$ in diameter, in chains of variable length; involution forms frequent.

Staining.—Gram-positive.

Culture.—Acrobe and facultative anaerobe; temperature range—generally $25^{\circ}-42^{\circ}\text{C.}$, optimum— 37°C. ; grows on ordinary media, but better on blood or serum media.

Agar stroke—growth consists of small circular discrete semi-transparent discs, about 1 mm. in diameter after twenty-four hours' incubation.

On blood-agar—larger colonies than on agar, clear zone develops round the colonies, due to diffusible haemolysin produced by the organism (*vide* p. 253).

Bouillon—growth forms as a granular sediment in the culture tube.

Biochemical Reactions.—Tested by growing in Hiss's medium with 1 per cent. of fermentable carbohydrate, and an indicator of acidity (litmus, Andrade's indicator, or neutral red, *vide* p. 104), or on serum-smear peptone-water-agar slopes containing the particular carbohydrate and an indicator (*vide* p. 96).

Glucose Lactose Saccharose Mannite Salicin Raffinose Inulin

⊥ ⊥ ⊥ ⊥ ⊥ - -

(⊥ = acid, no gas; - = no fermentation; ⊥ signifies that different varieties or types vary in the particular reaction)

Viability.—The thermal death-point is about 54°C. Cultures do not maintain their viability for any length of time, but survive best if kept at a low temperature—*e.g.* 0°C. Laboratory strains should be subcultured every four weeks.

OTHER TYPES OF STREPTOCOCCI OF MEDICAL IMPORTANCE

The streptococci constitute a somewhat heterogeneous group, and include types which are normal inhabitants of the mouth, throat and intestine.

These organisms have been broadly classified according to the appearance of growths on blood-agar. Thus in the case of *Streptococcus haemolyticus*, wide zones of haemolysis develop round the colonies (*vide supra*), and this is a characteristic feature. The designation " β type" has also been applied to this form of streptococcus.

A more reliable method of identifying a haemolytic streptococcus is to grow it in 20 per cent. serum broth for about fifteen hours and then to add varying amounts (*e.g.* 0.1 to 1 c.c.) of the culture to 0.5 c.c. of a 5 per cent. suspension of ox red blood corpuscles. The mixtures are incubated at 37° C. for one and a half hours, when haemolysis can be observed.

Certain varieties occurring as commensals in the mouth and throat are non-haemolytic and their colonies on blood-agar show a greenish coloration, due probably to peroxide formation and the resultant development of an oxidation product of haematin. It has been supposed also that this colour change is due to the production of methaemoglobin, but it is most marked when the organism is growing on medium containing heated blood. Streptococci producing such changes on blood media are designated *Streptococcus viridans*, or the " α (*alpha*) type."

A third group found as commensals in the mouth, throat and bowel produce no obvious change in blood media, and are sometimes designated the " γ type."

Morphological differences are of little significance in classification, though the faecal streptococci ("enterococcus") are usually oval in shape, or even lanceolate like a pneumococcus (*q.v.*), and occur in pairs or short chains.

Fermentative reactions have been extensively utilised for the differentiation of streptococci, but these cannot be regarded as sufficiently "fixed" characters for accurate classification. Certain biochemical tests, however, are of some significance in the general grouping of the streptococci. Thus the faecal streptococci are generally mannite- and aesculin-fermenters: the salivary types usually ferment raffinose.

A system of classification elaborated by Holman, and based on certain biochemical reactions along with haemolysis, has been extensively employed, and serves to differentiate the biological varieties met with. The following Table illustrates this classification:

	<i>Haemolysis</i>	<i>Fermentation of</i>		
		<i>Lactose</i>	<i>Mannite</i>	<i>Salicin</i>
<i>S. "infrequens"</i>	+	+	+	+
<i>S. "haemolyticus I"</i>	+	+	+	+
<i>S. pyogenes</i> ¹	+	+	+	+
<i>S. anginosus</i> ²	+	+	+	+
<i>S. "haemolyticus II"</i>	+	+	+	+
<i>S. "haemolyticus III"</i>	+	+	+	+
<i>S. equi</i> ³	+	+	+	+
<i>S. "subacidus"</i>	+	+	+	+
<i>S. faecalis</i> ⁴	+	+	+	+
<i>S. "non-haemolyticus I"</i>	+	+	+	+
<i>S. mitis</i> ⁵	+	+	+	+
<i>S. salivarius</i> ⁶	+	+	+	+
<i>S. "non-haemolyticus II"</i>	+	+	+	+
<i>S. "non-haemolyticus III"</i>	+	+	+	+
<i>S. equinus</i> ⁷	+	+	+	+
<i>S. "ignavus"</i>	+	+	+	+

¹ The classical type of pyogenic streptococcus. ² A pathogenic variety originally described in acute inflammatory conditions of the throat. ³ Of equine strangles. ⁴ The classical type of faecal streptococcus. ⁵ A commensal type found in the mouth and throat. ⁶ A mouth and throat commensal. ⁷ A type found in equine faeces.

Streptococci differ considerably in their resistance to heat, and this has been utilised as a basis of classification. Thus the faecal strains (enterococcus) are

able to withstand 60° C. for thirty minutes whereas this temperature kills the haemolytic streptococci and also the common mouth types.

The faecal streptococci are capable of growing on media containing bile-salt which is inhibitory to other types.

A capsulated streptococcus has been described—" *Streptococcus mucosus*." On blood-agar it produces slimy colonies with dark-green coloration of the blood; it has been noted in various suppurative conditions—e.g. otitis media.

Anaerobic (or microaerophilic) streptococci have been described. They correspond in certain of their biological characters to the aerobic forms, but haemolytic action is usually absent, and foetid gas formation

	<i>Haemolytic streptococci</i>	<i>Mouth and throat streptococci</i>	<i>Enterococcus</i> ¹ (<i>faecal streptococci</i>)
Morphology . . .	Chains of moderate length; cocci spheroidal	Chains of pronounced or moderate length; cocci often elongated	Usually pairs but may occur in short chains; cocci oval or lanceolate
Type as regards effect on blood (<i>vide supra</i>) . . .	β	α or γ	γ
Mannite-fermentation . . .	Usually -	-	+
Heat resistance (<i>vide supra</i>) . . .	-	-	+
Inhibition of growth by bile-salt . . .	+	+	-

¹ A certain proportion of enterococcus strains liquefy gelatin.

in culture is a common characteristic. These organisms have been found in septic lesions and in gangrenous conditions of mucous membranes. They are not infrequently present in puerperal sepsis and may produce a blood infection. They have also been found normally on mucous surfaces.

The Table on p. 255 summarises the main differential features of the three medically important groups of aerobic streptococci.

THE PATHOGENICITY AND OCCURRENCE OF STREPTOCOCCI

The pathogenic effects of the streptococci depend on the virulence of the particular strain and the susceptibility of the host. Thus a strain of low virulence may produce only a localised inflammation, whereas strains of high virulence may be associated with spreading inflammation and even septicaemia.

Haemolytic streptococci are found in the following conditions:—

Wound suppuration; localised abscesses; spreading inflammations—*e.g.* erysipelas, cellulitis, lymphangitis, lymphadenitis; septicaemia (*e.g.* puerperal septicaemia) and pyaemia; suppurative otitis and mastoiditis; meningitis, usually secondary to some other lesion—*e.g.* otitis; ulcerative endocarditis; inflammation of the fauces, tonsils, pharynx and larynx—*e.g.* acute follicular tonsillitis, the angina of scarlet fever (*vide infra*); broncho-pneumonia secondary to influenza, measles, etc.; peritonitis; urinary sepsis, etc.

In septic lesions, mixed staphylococcal and streptococcal infections are not infrequent.

Streptococci may also occur as secondary invaders—*e.g.* in diphtheria, etc.

Non-haemolytic types may occur as pathogens in lesions of a less acute form than those produced by the haemolytic varieties—*e.g.* cases of tonsillitis, otitis media, dental abscesses, broncho-pneumonia, cholecystitis. Subacute infective endocarditis is due to

this type of streptococcus. Even general infections of a septicæmic type may, however, be produced by these organisms.

Non-haemolytic streptococci isolated from the lesions of acute rheumatism have been designated "*Streptococcus rheumaticus*." Their relationship to the disease is doubtful.

SCARLATINA

Numerous observers have recorded the constant presence of large numbers of haemolytic streptococci in the throat in early cases of scarlet fever, and also that the majority of the complications of this disease are streptococcal, but it was not until 1923 that G. F. and G. H. Dick produced clinical scarlet fever by inoculating the throat of a volunteer with a culture of a haemolytic streptococcus isolated from the disease. The haemolytic streptococci associated with scarlet fever have been designated "*Streptococcus scarlatinae*." These observers later demonstrated that in the filtrates of fluid cultures of scarlatinal streptococci there is present a diffusible toxin which in suitable dilutions produces an erythema when injected intradermally into persons susceptible to the disease, while convalescents generally show no reaction. The test, now designated the *Dick reaction*, is made by injecting intradermally into the skin of one forearm 0.2 c.c. of a 1 : 1000 dilution of filtrate from a broth culture, while into the other forearm is similarly injected as a control a like amount of the same diluted filtrate which has previously been heated at 100° C. for at least one hour to destroy the toxin present. In a positive reactor there appears in six to twelve hours, at the site of injection of the toxin, a red erythematous patch usually about 2 cm. in diameter. The redness remains for about twenty-four hours and then slowly fades. The control injection—*i.e.* heated toxin—should produce only a transient redness just after

inoculation, and no erythema when readings of the test are made. For the reaction to be recorded as positive the diameter of the erythematous area should be at least 1 cm.; a typical positive usually shows an area 2-3 cm. in diameter. Pseudo-reactions, in which both toxin and control injections produce an erythematous reaction, may occasionally occur in adults, but such results are relatively uncommon in children. A positive Dick reaction indicates that the subject is sensitive to the streptococcal toxin, and that there is an absence of natural or acquired immunity. The reaction is positive in the early stages of scarlet fever, being visible over the redness of the rash, but becomes less intense as the disease progresses, and is usually negative after the third or fourth week of the disease. Convalescents from scarlet fever generally yield a negative reaction. It may be stated briefly that a positive Dick reaction shows susceptibility to scarlet fever, and an absence of immunity to the streptococcus toxin. Up to the present it has not been found possible to standardise streptococcus (Dick) toxin accurately as laboratory animals do not normally react to this product.

The toxin is prepared by growing a strain of *Streptococcus scarlatinæ* in Hartley's broth for forty-eight hours at 37° C. The culture is then centrifuged at high speed and the supernatant fluid decanted and passed through a tested earthenware (Berkefeld) or Seitz filter. The filtrate constitutes the "toxin." Phenol (0.5 per cent.) is added as a preservative, and for use the toxin is diluted 1 : 1000 with normal saline or preferably the buffer solution used for the reagents of the Schick test (*vide* p. 288).

It has been shown that serum from a convalescent scarlet fever patient, when injected intradermally in any early case of scarlet fever, causes a blanching or extinction of the rash around the site of injection. This is termed the *Schultz-Charlton reaction*, and shows that convalescent serum contains neutralising substances for the scarlet fever toxin. A similar result is obtained with antitoxic serum (*vide infra*).

This test may be employed clinically for diagnosis in doubtful cases of scarlet fever.

Active immunisation in susceptible individuals (*i.e.* Dick-positive) is carried out by injecting saline dilutions of toxin made up so that a certain volume—*e.g.* 1 c.c.—contains a given number of “skin-test” doses. Successive and increasing doses are given at weekly or fortnightly intervals, ranging from 500 to 80,000 “skin-test” doses. This treatment usually causes the Dick reaction to become negative.

By the immunisation of horses with streptococcus (Dick) toxin, an antitoxic serum is obtained which is used for therapeutic purposes.

The antitoxin is concentrated and refined in the same manner as diphtheria antitoxin (*q.v.*). It cannot be standardised accurately owing to the insusceptibility of animals to the toxin. The average dose of the refined product is 20 c.c., and if given intravenously reduces very considerably the toxic manifestations and incidence of complications, and shortens the stay in hospital. It should be noted that this antitoxin is effective in other conditions due to haemolytic streptococci such as erysipelas, puerperal fever, cellulitis, etc., and it is not necessary to have special antisera prepared from streptococci associated with these diseases.

The streptococci responsible for scarlatina are serologically heterogeneous. Griffith has recognised four different serological types, designated I, II, III and IV, which he states comprise 60 per cent. of strains, while the remainder form a heterogeneous group. There is, however, considerable overlap in antigenic composition among these serological types, and the four types referred to above may represent only a minority of the scarlatinal strains in particular outbreaks of the disease. It has been stated also that type IV is associated with a mild evanescent form of the disease after which a relapse due to another type may occur. The strain (“Dochez”) generally used for the production of Dick toxin and scarlatinal antitoxin belongs to the heterogeneous group.

THE OCCURRENCE OF STREPTOCOCCI IN DISEASES OF THE DOMESTICATED ANIMALS

S. haemolyticus is relatively infrequent in suppurative lesions of horses, sheep and swine, but may be met with more commonly in cattle. Conditions regarded as due to this organism are contagious pleuropneumonia of horses, omphalophlebitis of new-born animals with metastases in joints, "foot-rot" (a suppurative cellulitis) of sheep, acute mastitis of cattle, septicaemia of chickens (*S. gallinarum*).

Bovine mastitis.—The most prevalent organism in this condition is the streptococcus, and the type commonly found, especially in the chronic form of the disease, has been designated *S. agalactiae*: characterised as a long-chained streptococcus producing a flocculent type of growth in bouillon, acidifying and coagulating milk within forty-eight hours, fermenting glucose, lactose and saccharose but not acting on mannite; the strains are stated to vary in their haemolytic action on blood-agar, but it is doubtful whether the "haemolytic" strains are typical haemolysin-producers; the non-haemolytic strains are either of the α or γ type. Some strains of streptococci isolated from mastitis are indistinguishable from the true haemolytic forms found in pyogenic lesions of the human subject, and it has been assumed that such organisms are of human origin. The milk of cows suffering from such streptococcal infection may give rise to outbreaks of sore throat or scarlatina in the human subject, the type of streptococcus being typically haemolytic. This organism has sometimes been designated *S. epidemicus*.

Strangles of horses is due to a haemolytic streptococcus (*S. equi*) whose differential characters are indicated in Holman's classification (*vide supra*).

MICROCOCCUS TETRAGENUS (*Gaflkya tetragena*)

Morphology.—Spherical cocci in tetrads, about 0.7μ in diameter, and capsulated when growing in the tissues.

Staining.—Gram-positive.

Culture.—Aerobe and facultative anaerobe; optimum temperature— 37°C .; grows well on ordinary media.

Agar—growth resembles *Staph. albus*.

Gelatin—not liquefied.

Occurrence.—Suppuration in region of mouth and neck—e.g. dental abscess; cervical adenitis; pulmonary abscess, etc.

OTHER ORGANISMS ASSOCIATED WITH THE COMMONER SUPPURATIVE CONDITIONS

BACILLUS PYOCYANEUS (*Pseudomonas aeruginosa*)

Morphology.—Straight rods; 1.5 to 3 μ by 0.5 μ ; motile with one to three terminal flagella; non-sporing.

Staining.—Gram-negative.

Culture.—Aerobe and facultative anaerobe; temperature range—18°–43° C., optimum—30°–37° C.; grows on ordinary media.

Agar—stroke inoculation produces an abundant, moist, greenish-blue growth; the pigment (“pyocyanin”), on which the colour depends, also diffuses through the medium. Pigment is most abundantly produced at room temperature.

This organism forms also a yellowish-green fluorescent pigment named “fluorescein,” which is the characteristic product of an allied saprophytic organism, *B. fluorescens*.

Gelatin—liquefied, with a greenish-blue coloration.

Potato—growth is similar to that on agar but the pigment alters, as a result of oxidation, to a brownish colour.

Occurrence.—Suppurating wounds, usually in mixed infection with pyogenic cocci; otitis media, etc. This organism may occur as a commensal in the bowel of man and animals. In young subjects it may give rise to enteritis and may even produce a general infection. Organisms corresponding to *B. pyocyaneus* and *B. fluorescens* (*vide supra*) occur naturally in sewage, water and soil, and may also be found on the human skin.

BACILLUS PROTEUS (*Proteus*)

Morphology.—Straight rods about same size as *B. pyocyaneus*; pleomorphic; motile with lateral flagella; non-sporing.

Staining.—Gram-negative.

Culture.—Grows aerobically on ordinary media. Optimum temperature about 25° C., but grows well at 37° C. A single stroke inoculation on agar produces a moist, translucent, greyish-white growth, *which tends to spread all over the available surface of the medium*—hence the designation “spreader” often applied to this organism. Gelatin is liquefied. Coagulated serum is liquefied by some strains.

A non-motile variant type of *B. proteus* may be met with which does not spread on culture medium; it corresponds to the “O” variants of other motile bacteria (*vide* p. 344).

Biochemical Reactions.—*B. proteus* includes different biological types which vary somewhat in fermentative reactions:

Glucose	Lactose	Dulcitol	Saccharose	Mannite	Maltose	Indole formation	Serum liquefaction
+	—	—	±	—	±	±	±

(+ under carbohydrates signifies acid and gas production)

H₂S is formed, and urea is actively converted into ammonia.

Occurrence.—Septic infections — *e.g.* suppurating wounds, urinary sepsis, otitis media, etc.—usually along with other pyogenic organisms. It may be present in faeces, and occurs frequently as a saprophyte in decomposing organic matter. It has been reported as the causative organism in cases of acute bacterial food-poisoning.

In typhus fever the blood serum agglutinates a *B. proteus* type (“X 19”), originally isolated from the urine in this disease. This reaction is utilised as a diagnostic test (“Weil-Felix reaction”), but the organism has no aetiological relationship to the disease (*vide* p. 429). *B. proteus* strains of the “X 19” type are serologically different from other strains. This type also differs from other strains in the fermentation of salicin. The agglutinin responsible for the Weil-Felix reaction is of the “O” type (*vide* p. 34).

Another type designated “Kingsbury” is agglutinated by the serum of patients suffering from a typhus-like disease in Malaya, and is used in the diagnosis of this condition (*vide* p. 430). Salicin fermentation is absent (as compared with the “X 19” type).

**BACTERIOLOGICAL DIAGNOSIS IN PYOGENIC
INFECTIONS**

**COLLECTION OF SPECIMENS AND MICROSCOPIC
EXAMINATION**

Films are made on microscope slides from the pus or inflammatory exudate, dried and fixed by heat. *The films should, if possible, be made directly from the lesion.* For transmission to the laboratory the material is placed (by means of a pipette—*vide* p. 129) in a sterile stoppered test-tube. *Drying of pus intended for examination must be avoided*, and it is unsatisfactory to collect pus or discharges on cotton-wool swabs. In the case of *ulcers, sinuses*, etc., exudate may be taken with a sterile platinum loop, and films (and also cultures) are made at once.

In the case of *pleural and peritoneal fluids*, the fluid should be withdrawn into an equal volume of sterile citrate-salt solution (sodium chloride, 0·6 per cent., sodium citrate, 1·5 per cent.). This is to avoid coagulation, which renders cytological and bacteriological examination difficult. The material is centrifugalised and films are made from the deposit.

Urine should be drawn, with aseptic precautions, by means of a catheter lubricated with sterile oil. No antiseptics are to be used. The specimen is passed directly into a sterile stoppered bottle, and should be submitted for investigation without any delay. In the female this procedure is absolutely necessary to avoid contamination. In the male, however, a fairly satisfactory specimen may be obtained by cleansing the urinary meatus and, after a portion of the urine has been voided, collecting a sample directly in a sterile bottle. The urine is centrifugalised, and films are made from the deposit. After the films have been dried and fixed they should be gently washed in water to remove crystalline deposit.

Sputum should be expectorated directly into a

small sterile wide-mouth stoppered bottle. Films are made as in the case of pus.

Films are stained (1) by Gram's method; (2) with Löffler's methylene blue.

CULTIVATION

Successive stroke inoculations are made on nutrient agar (preferably blood-agar) in Petri dishes or sloped in tubes (*vide* p. 132). A proportion of the growth will be represented by separate colonies, and the colony characters of the organisms can be recognised. Films are also made from colonies and stained by Gram's method. In this way the organism present can generally be identified. If a mixed growth results, single colonies can be "picked off" on to agar or blood-agar slopes, so that pure cultures are available for any further examination required.

If *septicaemia* or *pyaemia* is suspected, blood culture is carried out (*vide* p. 141).

ORGANISMS SPECIFICALLY ASSOCIATED WITH CONJUNCTIVITIS

KOCH-WEEKS BACILLUS (*Haemophilus conjunctivitis*)

Associated with acute contagious ophthalmia.

Morphology.—Short slender rods about 1–1.5 μ in length; intracellular position in polymorph leucocytes of the inflammatory exudate is characteristic.

Staining.—Gram-negative.

Culture.—Does not grow on ordinary media but like *B. influenzae* (*q.v.*), to which it is closely allied, can be cultivated on media containing blood; in its growth requirements it is in fact similar to the influenza bacillus. On blood-agar, growth develops in the form of minute dewdrop-like colonies.

DIPLO-BACILLUS OF MORAX (*Haemophilus lacunatus*)

Associated with subacute or chronic conjunctivitis.

Morphology.—Rod-shaped organism measuring about 2 μ by 1 μ , in pairs end to end; non-motile.

Staining.—Gram-negative.

Culture.—Aerobe; requires blood or serum for growth; optimum temperature is about 37° C., and no growth occurs at room temperature; on solidified serum, growth produces liquefaction, and colonies develop “pits” or “lacunae” on the surface of the medium.

Diplo-bacillus liquefaciens (Petit) is morphologically similar to the bacillus of Morax, but grows well on ordinary nutrient media at 20°-37° C. It liquefies coagulated serum, but, unlike the other, grows in gelatin at 22° C. and liquefies it. It is associated with conjunctivitis in which there may be primary involvement of the cornea.

DIAGNOSIS

Films should be made from a loopful of conjunctival exudate. Stain with dilute carbol fuchsin, or by Gram's method. Make cultures on blood-agar. Pure cultures should be obtained from single colonies and the biological characters of the particular organism determined.

Other organisms found in conjunctivitis are: the gonococcus, pneumococcus, *Staphylococcus*, *Streptococcus*, organisms of *B. coli* group. *Staphylococcus albus* and diphtheroid bacilli --e.g. *B. xerosis* (q.v.)-- are frequent normal inhabitants of the conjunctival sac.

PNEUMOCOCCUS (*Diplococcus pneumoniae*)

The causative organism of acute lobar pneumonia.

Morphology.—The typical appearance is that of a lancolate or oval coccus in pairs with the rounded ends together; it is about 1 μ in its long diameter; shows a thick capsule, which appears as an unstained zone round the organism, unless positively stained by special methods (*vide* p. 163). In culture it is not so typical, being less lancolate and more rounded; the capsule is not so evident, and the cocci may occur in chains.

Staining.—Gram-positive.

Culture.—Aerobe; optimum temperature, about 37° C.; does not grow below 25° C.; grows on ordinary media, but best in the presence of blood or serum.

On agar—growth consists of small (about 1 mm.

diameter), delicate, semi-transparent, dewdrop-like colonies, which tend to remain discrete—*i.e.* like colonies of streptococci.

In bouillon—growth shows at first a uniform turbidity, but later forms a granular deposit in the tube.

On blood-agar—it is non-haemolytic, or only partially lytic, and the colonies produce the same greenish coloration as the “viridans” type of streptococcus. The colony is flat and smooth, and the edge is often distinctly raised.

Cultures may show transformation of the colonies from the typical smooth (S) type to the rough (R) form; this variation is associated with loss of capsule formation, absence of type-specificity (*vide infra*) and loss of virulence.

Biochemical Reactions.—Ferments various carbohydrates (*e.g.* glucose, lactose, saccharose) and differs from most strains of streptococci in its fermentation of inulin. Hiss’s serum water, containing 1 per cent. of peptone, is a convenient medium for these tests. The medium used for testing the biochemical reactions of the streptococcus also gives good results (*vide p.* 252). It is sharply differentiated from the streptococci by its solubility in bile. This test consists in adding 1 part of sterile ox bile or 1 part of a sterilised 10 per cent. solution of sodium taurocholate in normal saline to 10 parts of a bouillon culture. The test may also be carried out by using a 10 per cent. solution of sodium desoxycholate made distinctly alkaline to phenolphthalein, 0.1 c.c. being added to 1 c.c. of a broth culture. This method gives very satisfactory results, lysis occurring within fifteen minutes.

Viability.—The thermal death-point is about 52° C. The ordinary laboratory cultures lose viability rapidly and require to be subcultured at short intervals (*e.g.* about fourteen days). Cultures survive best in a semi-solid agar containing blood. The vitality and virulence of a strain can be maintained by the method described below.

Experimental Inoculation.—Pathological material containing pneumococci (*e.g.* pneumonic sputum) or a

virulent culture, injected subcutaneously into rabbits or mice, produces a rapidly developing septicaemia, fatal in twenty-four to forty-eight hours; at autopsy, typical capsulated diplococci are present in large numbers in the heart blood. The virulence for animals rapidly decreases if the organism is grown on media without blood. By drying the spleen of an infected mouse in a desiccator, the virulence of the organisms present may be maintained for a month or more. The spleen should be removed immediately after death.

Serological Types.—Four main types, classified I, II, III, IV, have been recognised, differing in their agglutination reactions. Types I, II and III are sharply defined from each other, and identified by their agglutination with specific type antisera. Pneumococci, which are not agglutinated by Type I, II or III sera, are all grouped together as Type IV. Type III is easily recognised also by its cultural characters; it is the "*Pneumococcus mucosus*," and produces raised mucoid or slimy colonies. Types I and II are the so-called "epidemic" types, being responsible as a rule for over 60 per cent. of cases of acute lobar pneumonia. Type IV (more correctly called Group IV) consists for the most part of individual strains which are not interrelated, and the pneumococci normally occurring in the mouth and throat as commensals belong mostly to this type. The determination of the particular type of pneumococcus is important from the therapeutic aspect, as antisera (horse) for Type I, or for both Types I and II, are available for the treatment of infections due to these types.

The identification of the type is carried out by using a pure culture of the pneumococcus, and known type sera (I, II, III). For ease and rapidity of obtaining a suspension of the organism, advantage is taken of the extreme susceptibility of the mouse (*vide supra*). The animal is inoculated intraperitoneally with 0.5–1 c.c. of a saline emulsion of sputum (or any other pneumococcal exudate). The mouse may die in seven to twenty-four hours after injection; the peritoneal cavity is then opened with aseptic precautions, and the exudate is examined

microscopically. If abundant pneumococci are present, the peritoneal cavity is washed out with 5 c.c. sterile saline solution and cultures are made on blood- or serum-agar plates. The peritoneal washings are centrifuged at low speed to deposit fibrin and cells. The supernatant fluid is removed and centrifuged at high speed to throw down the organisms. The bacterial sediment is then emulsified in saline solution to form a moderately dense suspension. The test is carried out as follows¹:—

Tube 1.	0.5 c.c.	Serum Type I (1:20)	² + 0.5 c.c. bacterial suspension
Tube 2.	0.5 c.c.	„ Type II (undiluted)	+ 0.5 c.c. bacterial suspension
Tube 3.	0.5 c.c.	„ Type II (1:20)	+ 0.5 c.c. bacterial suspension
Tube 4.	0.5 c.c.	„ Type III (1:5)	+ 0.5 c.c. bacterial suspension
Tube 5.	0.1 c.c.	{ 10 per cent. sodium } taurocholate or sodium desoxycholate } 0.4 c.c. bacterial suspension	

Tube 5, containing bile salt *plus* bacterial suspension, determines the bile solubility of the strain and excludes streptococci. The tubes are incubated for one hour, and those showing agglutination after that time are noted.

The following Table shows the results of determining pneumococcus types by this method:—

Pneumococcus Suspension 0.5 c.c.	Serum I (1 in 20) 0.5 c.c.	Serum II (undiluted) 0.5 c.c.	Serum II (1 in 20) 0.5 c.c.	Serum III (1 in 5) 0.5 c.c.	10 per cent. Bile salt 0.1 c.c. + pneumo- coccus suspension 0.4 c.c.
Type I . . .	++	—	—	—	+
Type II . . .	—	++	++	—	+
Type II (atypical) . . .	—	+	—	—	+
Type III . . .	—	—	—	++	+
Type IV . . .	—	—	—	—	+
Streptococcus . . .	—	—	—	—	—

+ indicates the degree of agglutination, and in column 5, bile solubility.

¹ See technique of agglutination tests (p. 196).

² The optimum dilutions for the test may vary somewhat with different sera and should be determined by preliminary tests with known pneumococcus strains.

It is to be noted that atypical Type II organisms are agglutinated only by undiluted Type II serum. A Type IV pneumococcus shows no agglutination with any of the sera, but is bile-soluble, whereas streptococci are neither agglutinable nor bile-soluble.

Rapid Method.—When it is necessary to ascertain the type with the least delay, some peritoneal fluid may be aspirated by means of a syringe after four or five hours. Four small drops of the fluid are placed on a slide and mixed with equal drops of 1 in 10 dilutions of antisera for Types I, II and III, and of saline respectively. The mixed drops are each spread in the form of films, fixed and stained. On microscopic examination specific agglutination may sometimes be observed and the type of pneumococcus identified. If the result is negative or doubtful, the peritoneal exudate can be examined later in the usual way.

Direct Method of typing the Pneumococcus.—It has been shown that when the pneumococcus, present in sputum from a case of pneumonia, is acted on by a specific serum it becomes swollen and shows a clear refractile capsular zone. This reaction has been utilised as a direct method of typing. The procedure is as follows: an emulsion of the sputum is prepared in physiological saline solution and four drops of this emulsion, deposited on slides, are mixed with corresponding drops of the three type antisera and of saline respectively, the last serving as a control. The mixtures are covered with No. 1 cover-slips and examined with an oil-immersion lens, the substage condenser being suitably lowered and the diaphragm reduced in aperture. The enlargement of the pneumococci in the presence of the particular type serum can readily be observed.

For further details of this method, see R. R. Armstrong, 1932, *Brit. Med. Journ.*, vol. 1, p. 187, and W. R. Logan and J. T. Smeall, 1932, *Brit. Med. Journ.*, vol. 1, p. 188.

Specific Substances of the Pneumococcus.—Type specificity is dependent on polysaccharide haptens (*vide p. 29*) contained in the capsule of the organism. Loss of the capsule-forming property and R transformation of the colonies (*vide supra*) are associated with absence of the specific carbohydrate and of type-specificity and also loss of virulence.

Occurrence.—In lobar pneumonia pneumococci are present in large numbers in the consolidated areas, and can easily be detected in the sputum. They can be

demonstrated in the blood by blood culture, and also occur in the pathological complications of pneumonia—*e.g.* pleurisy, empyema, endocarditis, pericarditis, meningitis, arthritis, etc.

The pneumococcus is found in broncho-pneumonia, simple catarrhal conditions of the throat and respiratory tract, conjunctivitis, otitis media, meningitis, primary peritonitis.

As noted above, this organism may be present normally in the mouth and throat secretions.

DIAGNOSIS

A specimen of sputum is obtained (*vide* p. 263), and films are stained by Gram's method. Cultures are made by successive strokes on a blood-agar plate. The characteristic colonies can be recognised among the other organisms that constantly develop from sputum, and are picked off on to blood- or serum-agar slopes for purposes of isolation. The fermentation of inulin and bile-solubility are determined with the pure cultures obtained.

The occurrence of septicaemia with characteristic diplococci in the heart blood on inoculation of a mouse with the sputum or the culture isolated, is conclusive proof of the identity of the organism present, even if the diplococci noted in the specimen or cultures are not entirely typical.

The serological type can be determined by the methods described above.

*Lung Puncture.*¹—In cases in which no sputum is obtainable—*e.g.* at an early stage of the illness—and it is necessary to ascertain the serological type of pneumococcus present, lung puncture may be resorted to. A fine-bore exploratory needle attached to a Record syringe is used. The needle is introduced directly into the lung substance for 8 to 10 cm. and the piston is slowly withdrawn. A few drops of blood-stained fluid can thus be aspirated. This is mixed with 5 c.c. of broth in a tube and 1 c.c. of the mixture is injected into a mouse as in dealing with sputum for serological typing of the organism (*vide supra*).

¹ See Stewart, *Lancet*, 1930, ii., p. 520.

CHAPTER X

MENINGOCOCCUS; GRAM-NEGATIVE COCCI OCCURRING IN THE NOSE AND THROAT; GONOCOCCUS

MENINGOCOCCUS (*Neisseria intracellularis*)

CAUSATIVE organism of epidemic cerebro-spinal meningitis.

Morphology.—Oval diplococci with opposed surfaces flattened or concave; sometimes in tetrads; cocci are about $0.8 - 1 \mu$ in diameter; the long axes of the cocci in pairs are parallel, not in line as in the case of the pneumococcus (*q.v.*). In cerebro-spinal fluid the intracellular position in polymorph leucocytes is characteristic. In culture the characteristic shape and arrangement seen in the spinal fluid may be lost, and involution forms are noted.

Staining.—Gram-negative.

Culture.—Aerobic conditions are necessary for satisfactory growth; temperature range is $25^{\circ} - 42^{\circ} \text{C.}$, and the optimum is about 37°C. ; requires blood or serum for growth. A specially suitable medium is a nutrient agar prepared from a digest basis, and containing 5 per cent. blood added to the melted agar at 90°C. (*vide* p. 108).

Colonies on serum-agar—after twenty-four hours are small, greyish, transparent, circular discs about 2 mm. in diameter—*i.e.* larger than colonies of streptococci; later the centre of the colony becomes more opaque and raised, while the periphery remains thin and transparent; the borders may become radiate and crenated. While this is the common type of colony, considerable variation in the colony appearances may be noted.

Colonies on blood-agar are like those on serum-agar but somewhat larger; they are smooth, grey and semi-transparent; no haemolysis occurs.

Biochemical Reactions.—Can be tested by growing on peptone-water-agar slopes containing 5 per cent. serum, 1 per cent. of the particular sugar, and an indicator (*vide* pp. 96, 252).

Ferments glucose and maltose with acid production, but has no action on lactose, saccharose, or inulin.

Viability.—When first cultivated artificially the meningococcus tends to die out quickly in culture—*e.g.* within two or three days. In culture it persists best at incubator temperature. Cultures remain viable for a longer time on an egg medium. The meningococcus is very sensitive to drying, and when desiccated dies within two hours.

Serological Types.—Gordon recognised two main types (Types I and II) differentiated by agglutination and agglutinin-absorption reactions with antisera, and representing over 80 per cent. of all cases occurring in this country. Other less frequent serological types (*e.g.* III and IV) were also identified.

These types are not so sharply differentiated as the types of the pneumococcus, and Griffith has classified strains into two serological groups, one of which includes Gordon's Types I and III, and the other, Types II and IV. He has also shown that the antigenic structure of individual strains may be complex and made up of multiple constituents.

Agglutinating antisera are prepared by immunising young rabbits with successive and graded doses of dead cultures (*vide* p. 202) injected intravenously. The initial dose should be 500–1000 million organisms. The serum of the immunised animal may agglutinate the homologous organism in dilutions up to 1 in 400 or 1 in 800. In carrying out the agglutination test, the general method described in Chapter VII. may be followed, but the tubes should be placed in a water-bath at 55° C. for twenty-four hours, when the results can be read.

DIAGNOSIS

In the early stages of cerebro-spinal meningitis the organisms are present usually in considerable numbers in the cerebro-spinal fluid, and can be recognised by

microscopic examination. At a later stage they may be scanty and even apparently absent.

The specimen of fluid is obtained by lumbar puncture with a special nickel or platinum-iridium needle fitted with a stilette. The patient is anaesthetised if necessary, and placed on the right side with the knees drawn up and the left shoulder thrown forward. The skin over the lumbar region, the operator's hands and the needle are sterilised by appropriate methods, and a sterile stoppered tube should be ready for collection of the fluid. The puncture is made in the middle line between the 3rd and 4th, or 4th and 5th lumbar vertebrae, the needle being inserted, with the stilette in position, in a forward and slightly upward direction. In the adult it is introduced to a depth of about 5-6 cm. The stilette is withdrawn, and the fluid is allowed to flow into a test-tube. In a case of cerebro-spinal meningitis the spinal fluid is under pressure and turbid in appearance, due to the large number of pus cells present.

In the laboratory the fluid is centrifugalised, and films are made from the sediment and stained by (1) methylene blue, (2) Gram's method. Cultures should also be made on one of the blood or serum media referred to above. Films are made from the resulting colonies or growth and stained by Gram's method. The colony characters should be ascertained, and subcultures for further tests are obtained by picking off single colonies on to blood- or serum-agar slopes. The biochemical reactions can be tested and, if necessary, the serological group identified by agglutination tests with antisera to the various recognised types.

For clinical diagnosis the microscopic examination is generally sufficient — *i.e.* if Gram-negative, intracellular diplococci with the characteristic shape of the meningococcus are observed. While meningococci are present in large numbers usually at an early stage of the illness, they may become relatively scanty in the cerebro-spinal fluid at a later stage and even undetectable by microscopic methods. They may be

demonstrated by cultural methods when difficult to find by microscopic examination. A method which sometimes facilitates the cultivation of the organism is to add an equal volume of glucose-broth to the cerebro-spinal fluid and incubate the mixture for eighteen hours; thereafter subinoculations are made on a solid medium as described above.

In carrying out routine investigations of cases of meningitis, other causal organisms must be considered—e.g. pneumococcus, tubercle bacillus, etc. If no organisms are detectable in films stained by methylene blue or Gram's method, a Ziehl-Neelsen preparation should be examined for tubercle bacilli (*vide* p. 301). In tuberculosis the cell exudate is mainly lymphocytic as compared with the polymorph exudate in meningococcal and pneumococcal meningitis. In young children, acute meningitis may be caused by a haemophilic bacillus, similar in type to *B. influenzae* (*q.v.*). In the cerebro-spinal fluid this organism usually develops elongated filaments, and these are seen among the polymorph leucocytes of the exudate. It is also to be noted that the virus of poliomyelitis may produce inflammatory conditions of the meninges, indicated by the presence of a leucocytic exudate in the cerebro-spinal fluid. In such cases no visible organisms are present.

DIAGNOSIS OF MENINGOCOCCUS CARRIERS

During an epidemic of cerebro-spinal meningitis healthy contacts may become carriers, and meningococci are found in the naso-pharynx. The recognition of such carriers is of importance in controlling the spread of the disease, and this depends on culturing the meningococcus from the naso-pharyngeal secretion.

Cultures should not be made within an hour after a meal, or within twenty-four hours after the application of antiseptics to the throat.

The specimen is best obtained by means of a swab (*vide* p. 290), with a longer wire-holder than the usual

throat swab, and with the terminal $\frac{3}{4}$ -in., carrying the cotton-wool pledget, bent through an angle of about forty-five degrees. The swab with wire-holder is enclosed in a stoppered test-tube of sufficient width to admit the bent end. It is necessary that cultures should be made immediately after swabbing and the medium incubated at once, owing to the feeble viability of the organism apart from the body.

The tongue is depressed, and the swab is passed behind the soft palate and introduced into both posterior nares. Before withdrawal, the swab is also rubbed over the posterior wall of the naso-pharynx. The swab must be introduced and removed from the mouth without touching the tongue.

West's Post-Nasal Swab is specially adapted for the purpose; it consists of a curved tube containing a flexible wire with a wool pledget as in the case of a throat swab. The tube is introduced into the mouth, and by means of its curve can be passed up behind the palate; from this end the swab is protruded into the naso-pharynx. It can then be retracted, and the tube is withdrawn from the mouth.

The swab is rubbed at once over a small area at the edge of a serum- or blood-agar plate already prepared, and then successive stroke inoculations are made on the remainder of the plate by means of a platinum loop, the loop being charged several times from the area inoculated directly with the swab. It is advisable to use agar containing a digest basis. The plate must be incubated without delay.

Suspicious colonies are examined by means of Gram-stained films, and subcultures from single colonies are made on serum- or blood-agar slopes.

The resulting pure cultures are then available for identification. The differentiation of other Gram-negative throat diplococci from the meningococcus is considered below. The absence of growth at room temperature, the inability to grow on ordinary medium, the sugar reactions, and the readiness with which the growths emulsify in saline, are important features in the identification of the meningococcus.

The final identification is carried out by means of a polyvalent agglutinating anti-meningococcus serum or antisera to the different types.

THE GRAM-NEGATIVE DIPLOCOCCI OCCURRING AS COMMENSALS IN THE NOSE AND THROAT

DIPLOCOCCUS CATARRHALIS (*Neisseria catarrhalis*)

A frequent commensal in the throat and nose, and often regarded as pathogenic in catarrhal inflammations of the respiratory tract.

Morphology and Staining.—Practically identical with the meningococcus.

Culture.—Grows on ordinary media without serum; grows at room temperature; the colonies are usually larger than those of meningococcus, especially when fully grown, thicker and more opaque, and exhibit a somewhat tough consistence, so that they can actually be moved about on the medium with a platinum loop without being broken up. The colony characters may vary considerably from the features described above. The organism exhibits no fermentative properties (*vide infra*).

D. catarrhalis is not agglutinated by meningococcus antisera.

DIPLOCOCCUS FLAVUS TYPES (e.g. *Neisseria flava*)

The *morphology* of these organisms is like that of *D. catarrhalis*, and they grow on ordinary media at room temperature. Cultures develop, after forty-eight hours, greenish-yellow or greenish-grey colours.

Young colonies may simulate closely those of the meningococcus.

Biochemical reactions, which vary according to the type, are shown in the Table on p. 277.

They are not agglutinated specifically by meningococcus antisera, but may agglutinate in low dilutions of horse serum. This has to be remembered in identifying meningococci from carriers when antisera from horses are used for the serological test.

DIPLOCOCCUS PHARYNGIS SICCUS (*Neisseria sicca*)

Resembles *D. catarrhalis*, but its colonies are markedly dry, tough and adherent to the medium. It seems possible that this organism is not a separate species, but a "rough" variant of some other member of the group.

Biochemical Reactions.—See Table.

DIPLOCOCCUS MUCOSUS

Differs from the other members of the group in being capsulate and producing mucoid colonies.

DIPLOCOCCUS CRASSUS

Resembles *D. catarrhalis*, but shows marked variation in its staining by Gram's method, some individual cells staining Gram-positive, others Gram-negative. The colonies are small and rather like those of the streptococci. Growth occurs at room temperature.

Biochemical Reactions.—See Table.

NEISSERIA FLAVESCENS

This organism has been described as the causative organism in a group of cases of meningitis in America. It resembles the meningococcus in morphology, but on blood-agar produces golden-yellow colonies. It does not ferment carbohydrates.

FERMENTATIVE REACTIONS OF GRAM-NEGATIVE DIPLOCOCCI

	<i>Glucose</i>	<i>Lactose</i>	<i>Saccharose</i>	<i>Maltose</i>
<i>Meningococcus</i> .	⊥	-	-	⊥
<i>Gonococcus</i> .	⊥	-	-	-
<i>D. catarrhalis</i> .	-	-	-	-
<i>D. flavus</i> types .	⊥	⊥	⊥	⊥
<i>D. pharyngis sicca</i>	⊥	-	⊥	⊥
<i>D. crassus</i> .	⊥	⊥	⊥	⊥

(⊥ = acid; ⊥ = variation in reaction among different types)

GNOCOCCUS (*Neisseria gonorrhoeae*)

The causative organism of gonorrhoea.

Morphology.—Oval diplococci with opposed surfaces flattened or even concave. The diameter of the coccus is about $0.8-1\mu$. Morphologically the gonococcus is identical with the meningococcus. In inflammatory exudates the intracellular position of the organism is characteristic, and pus cells often appear to be almost filled with ingested diplococci. In culture, involution forms are frequent.

Staining.—Gram-negative.

Culture.—Aerobe; temperature range— $30^{\circ}-39^{\circ}\text{C.}$, optimum— $36^{\circ}-37^{\circ}\text{C.}$; requires blood or serum for growth—*e.g.* agar containing 5–10 per cent. blood (heated at 55°C.), serum-agar prepared from fresh sterile serum (*vide* p. 105), or agar containing 10 per cent. ascitic fluid. Various special media have been recommended, but the above-mentioned serve satisfactorily in the routine cultivation of the organism. The agar should be carefully standardised to pH 7.5.

Care should be taken to ensure that the medium used is sufficiently moist and is kept moist during incubation. For this purpose plate cultures may be incubated inside a closed jar containing a pad of cotton-wool moistened with water.

Colonies are semi-transparent discs about the size of a pin head, tending to remain discrete, circular in outline at first, but later showing a “scalloped” margin, a raised, more opaque centre, and sometimes radial and concentric markings. Papillae may be noted after some days’ growth.

Two different types of colonies have been described: a large, thin irregular colony on which papillae develop and a small round colony which is more raised and without papillae.

In primary cultures, colonies may be slow in developing and growth may not appear for three or four days.

Biochemical Reactions.—Can be tested for as in the case of the meningococcus, fresh serum being added

to the medium used; ferments glucose, but not maltose (*vide* Table *supra*).

Viability.—The thermal death-point is about 55° C. This organism is very susceptible to drying, and when desiccated dies within two hours. It is a strict parasite and dies rapidly (*e.g.* in one or two hours) when discharged from the body, especially if subjected to cooling and drying. When first cultivated, cultures have a feeble viability, and subcultures should be made every three or four days to maintain the strain. When accustomed to artificial growth, cultures survive longer if kept at 37° C., and in a moist condition—*e.g.* two to three weeks. Cultures die at room temperature in two days.

Occurrence.—In the *male* the gonococcus infects the mucosa of the urethra and produces a suppurative inflammation with purulent discharge. Gonococci are present in large numbers in the discharge at an early stage, but later diminish, and are then associated with secondary infecting organisms—*e.g.* pyogenic cocci, *B. coli*, diphtheroid bacilli. They may invade the prostate, vesiculæ seminales, epididymis, bladder mucosa, and peri-urethral tissue (producing a peri-urethral abscess).

In the *female* the urethra and cervix uteri are infected, but rarely the vaginal mucosa. Bartholin's glands, the endometrium, and the Fallopian tubes may be invaded, and even the peritoneal cavity.

Purulent conjunctivitis may occur as a complication. Blood invasion may result from primary gonorrhoeal infections, and arthritis is a common complication. Ulcerative endocarditis has been noted as an occasional sequela.

In female infants and children, the gonococcus may produce a persistent vulvo-vaginitis.

In new-born infants gonorrhoeal ophthalmia may result from direct infection at birth.

DIAGNOSIS

Films are made from the discharge.—In the *male*: from the urethral discharge; the meatus should be cleansed with sterile gauze soaked in saline solution,

and specimens are taken either with a platinum loop, or directly on slides. In the *female*: from the urethra and cervix uteri, using a platinum loop and with the aid of a vaginal speculum.

The films are stained by (1) methylene blue, and (2) Gram's method (using neutral red as the counter-stain), and in the acute stage, both in the male and female, the occurrence of the characteristic *Gram-negative intracellular* organisms is, for clinical purposes, diagnostic.

In *Chronic infections*, particularly in the female, the cocci may be relatively scanty in films, and difficult to identify accurately among the secondary infecting organisms. In the male the "morning drop" of secretion from the urethra should be examined, or films from the centrifuged urinary deposit or the discharge after prostatic massage. In the female the secretion from the cervix uteri should be examined.

The diagnosis may be confirmed by cultivation, but where there is a mixed infection this may be technically difficult. Inoculation with material to be cultivated should, if possible, be made directly from the patient on a suitable medium (*vide supra*), and the culture should be incubated at once. If the material is kept at room temperature for some time before inoculation and incubation, or if it is allowed to dry, the organisms, being strict parasites, may die and fail to grow on the culture medium.

Serum Diagnosis.—The complement-fixation test is applicable for diagnosis and may be of considerable value to the clinician. Various types of antigen prepared from cultures of the gonococcus have been used. The antigen recommended is that devised by Price¹ who claims that in the first week of the disease 27 per cent. of positive results are obtained, rising to 46, 70, 89 and 100 per cent. after two, three, four and five weeks respectively.

This antigen is made as follows: gonococci are grown on

¹ I. N. O. Price. Monograph published by the London County Council, No. 2995, 1933; see also *Journ. Path. and Bact.*, 1932, vol. 35, p. 635.

hydrocele agar in a Roux flask at 37° C. for twenty-four to forty-eight hours. They are then washed off into a cylinder with 100 c.c. saline solution. 1 c.c. of N/1 caustic soda is added and the cylinder placed in a 37° C. water-bath for two hours, when most of the organisms will have become dissolved. The fluid is filtered through sterile lint to remove any gross insoluble matter. To the clear filtrate is added 1·5 c.c. N/1 HCl and the cylinder is replaced in the water-bath. White flocculi appear after ten to twenty minutes, and these are centrifuged at high speed and the deposit suspended in 4 c.c. normal saline. N/10 caustic soda is added to the suspension drop by drop until pH 7·5 is reached. After vigorous shaking the suspension undergoes solution. 1 c.c. of 1 per cent. formol-saline is now added and the fluid is filtered through lint. This solution constitutes the gonococcal antigen and is used in the test in a dilution of 1 in 30.

The general technique of the test is very similar to the Wassermann test described on p. 215.

CHAPTER XI

BACILLUS DIPHTHERIAE AND BIOLOGICALLY ALLIED ORGANISMS; B. PSEUDO-TUBERCULOSIS OVIS AND BACILLUS OF EQUINE ULCERATIVE LYMPHANGITIS; BACILLUS PYOGENES

BACILLUS DIPHTHERIAE (*Corynebacterium diphtheriae*, *Klebs-Löffler bacillus*)

THE causative organism of diphtheria.

Morphology and Staining.—Slender rod-shaped organism, straight or slightly curved; the average size is 3μ by 0.3μ , but longer and shorter forms may be noted; the ends are often swollen; it is non-motile and non-sporing. In culture, involution forms may be observed which are pear-shaped, club-shaped, or even globular. The bacillus is Gram-positive, though more readily decolorised than many other Gram-positive organisms; stained with methylene blue it shows marked "beading," and by Neisser's method (*vide* p. 160) metachromatic granules are characteristic, staining blue-black in contrast with the light-brown coloration of the rest of the organism; the granules are often polar in situation. If over-decolorised in the Gram method the granules tend to retain the violet stain, while the rest of the organism is decolorised. These characteristic staining reactions may be elicited in culture only when the bacillus is grown on a serum medium.

Culture.—Aerobe; temperature range— 20° – 40° C., optimum— 37° C.; grows on ordinary nutrient media, but best on serum media.

Colonies on Löffler's serum—at first small, circular, white, opaque discs with regular borders; later the centre becomes thicker and the borders crenated; they may reach in diameter 4–5 mm. after several days' growth; sometimes the growth on serum shows a distinct yellow tint.

The characters of colonies on the tellurite medium specially used for the isolation of *B. diphtheriae* are described on p. 114.

Does not liquefy gelatin.

In bouillon—grows in small white masses, which settle in bottom of tube and also adhere to side of tube; a surface film of growth may also develop.

Biochemical Reactions.—Ferments with acid production, glucose, galactose, maltose and dextrin, but not lactose, saccharose or mannite. Some strains ferment starch and glycogen.

These reactions can be elicited by using Hiss's serum medium (*vide* p. 104).

Certain workers have considered that the strains which ferment starch and glycogen are responsible for the more severe types of diphtheria, and have named them "*B. diphtheriae gravis*," while the non-fermenting strains termed "*B. diphtheriae mitis*" are said to be associated with the milder type of the disease. These views have not been confirmed by other observers.

Viability.—In culture, diphtheria bacilli may remain alive for two or more months at room temperature. In the moist condition they are comparatively easily killed by heat (in ten minutes at 60° C.), but when dry survive for much longer periods. The bacilli in dried membrane can resist 98° C. for one hour, and at room temperature and in the dark may remain alive and virulent for several months.

Occurrence.—The bacilli are present in large numbers in the "false membrane" and in the throat secretions. They do not invade the lymphatics to any extent, and there is no general blood infection. In nasal diphtheria the organisms can be detected in the nasal discharge. Infection of wounds, the conjunctiva, vulva and vagina may occasionally occur.

Diphtheria Toxin.—The diphtheria bacillus produces a powerful extracellular toxin with specific toxic properties. While the bacillus remains localised at the site of infection, the diffusible toxin is absorbed into the blood stream and leads to the various systemic disturbances of diphtheria and to the sequelae—*e.g.* post-diphtheritic paralysis.

When diphtheria bacilli are grown in suitable fluid media abundant toxin is produced. The strain used is however of considerable importance, for in artificial culture the organism may not adapt itself as readily to toxin production as it does in the human body. A single strain (Park-Williams No. 8) is almost universally used for toxin production on a large scale. The bacilli are removed from the culture by filtration or other means, and the bacteria-free liquid, which contains the exo-toxin mixed with the culture medium and other products of bacterial growth, is referred to as “toxin.”

Certain cultural conditions are required for the maximum production of toxin. An abundant supply of oxygen and a definite concentration of certain commercial peptones or of meat digested by either pepsin or trypsin are essential. In addition to nitrogenous materials, carbon-containing substances such as sodium acetate, sodium lactate, or maltose greatly enhance the toxicity of the filtrates. The use of maltose for this special purpose is of interest, because the maltose is only hydrolysed slowly into glucose by the intracellular enzyme maltase present in the organism. The presence of too high a concentration of glucose would lead to excessive acidity of the medium, and would be detrimental to the production of a strong toxin. In recent years it has been found possible by careful control of the medium and the conditions of growth to produce toxin ten times the strength of that obtained a few years ago. Hartley's broth (p. 83), or preferably the modification described by Pope and Smith (p. 116), is a very suitable medium, the cultures being made in shallow layers in conical flasks. When large-scale production is required it is usual to employ bottles of 5-litre capacity (Double W.Q.), containing 500 c.c. of medium and incubated in the horizontal position. Cultures are usually grown for ten days at 34°–35° C.

The fluid is then decanted from the sediment, covered with

toluol to kill the organisms and preserve the material, and kept in the cold for two or three days, being shaken occasionally. Alternatively the culture may be filtered through an earthenware or Seitz filter, and the bacteria-free filtrate constitutes the toxin.

The toxic substance so produced is unstable and its potency diminishes on exposure to air and light. In sealed tubes and in the dark it may remain unaltered for several weeks. The loss of toxic action is due to the conversion of toxin into "toxoid," which has no pathogenic effect on animals, but still retains the power of combining with antitoxin and of stimulating immunity.

This process of conversion of toxin into toxoid can be carried out by adding 0·3 per cent. of formalin and incubating the toxin for two to three weeks at 37° C. The change from the toxic into the non-toxic state is determined by injecting the material into guinea-pigs; when 5 c.c. injected subcutaneously or intraperitoneally produce no symptoms, the change is regarded as complete. Toxoid produced through the action of formalin in this way is a valuable immunising agent.

Diphtheria toxin is a powerful poison, particularly for guinea-pigs, in which 0·00025 c.c. may be fatal within five days. At the site of inoculation there is a greyish necrotic focus surrounded by an area of congestion, while the subcutaneous tissue shows marked inflammatory oedema. The neighbouring lymph glands are swollen and congested, the suprarenals are enlarged, very congested and may show haemorrhages; the lungs are usually congested and there is effusion into the pleural cavities; the kidneys and liver show degenerative changes. The amount of toxin formed varies with the strain of organism used and the composition of the medium, and each batch of toxin has to be standardised by animal experiment.

The same pathogenic effects as those described above are produced by the subcutaneous injection of a living culture of virulent diphtheria bacilli. The organisms remain more or less localised at the site of inoculation, while their toxin is absorbed into the circulation.

Virulence.—The microscopical appearance of *B. diphtheriae* gives no indication of the virulence of the organism, and when bacilli morphologically resembling *B. diphtheriae* are found in the throat or nose unassociated with active disease (*i.e.* in carriers) it is important to determine whether they are virulent or not. The virulence test is done by the intradermal injection of a culture into guinea-pigs as follows.

The primary cultures from the nose or throat are plated out and a single colony subcultured to obtain a pure growth. Serum-agar may be used but the tellurite-trypsin medium described on p. 113, McLeod's medium (p. 115), or Horgan and Marshall's blood-tellurite medium (p. 116) are recommended for the easy isolation of the diphtheria bacillus. The fermentative powers of the selected pure growth are tested, and if the organism ferments glucose, but not saccharose, a suspension from a serum-agar culture is made in saline of such a strength that the fluid is distinctly opalescent (about 500,000,000 bacilli per c.c.). Two white guinea-pigs of about 400 gm. weight are selected, and the hair removed from the flanks as described on p. 184. The day before the test is carried out, one of the animals—the control guinea-pig—is injected intraperitoneally with 1000 units of diphtheria antitoxin (*vide infra*). For the actual test each guinea-pig is injected intradermally with 0.2 c.c. of the suspension of organisms, using a 1 c.c. syringe and fine-bore needle (26 gauge, $\frac{3}{8}$ in. long). Several different cultures may be tested on each guinea-pig, and the injections should be about one inch apart. A careful note of the position of the injection of each different organism should be made. It is not advisable to carry out more than ten simultaneous tests on an animal. Four hours afterwards the test guinea-pig (*i.e.* the one that did not previously receive antitoxin) is now injected with 100 units of antitoxin. Alternatively the test guinea-pig may be injected immediately with $\frac{1}{50}$ th unit of antitoxin per gram of body weight. The guinea-pigs are examined

twenty-four, thirty-six and forty-eight hours after inoculation.

Virulent diphtheria bacilli produce in the test animal a well-defined red area about 15 mm. in diameter. After the third or fourth day the colour fades, leaving a necrotic patch with a scab surrounded by growing hair. The control guinea-pig shows no such reaction, the puncture wound caused by the needle being generally the only evidence of injection. If the organism is non-virulent there is no reaction in either the test or control animal. A reaction in both animals shows that the organism is not the diphtheria bacillus, because the products of growth are not neutralised by diphtheria antitoxin, as evidenced by the reaction in the control animal.

Diphtheria Antitoxin.—By immunising horses with toxin, an antitoxin is produced from the tissues and appears in the blood; the serum of such animals constitutes “diphtheria antitoxin.” For regulating the dosage of this antitoxin, an arbitrary “immunity unit” has been adopted and the number of such units in a given volume of serum denotes its antitoxic value. The unit as *originally* defined is that amount of antitoxin (or antitoxic serum) which just neutralises 100 M.L.D. of a certain toxin, the M.L.D. (minimum lethal dose) being the minimum amount which kills a guinea-pig of 250 grams weight in four days. It has not been feasible to preserve a standard toxin for testing antitoxin, but by means of a preserved standard antitoxin any toxin preparation can be standardised by neutralisation tests in guinea-pigs, and in turn the value of a new antitoxin can be estimated. The usual method for the purpose is to ascertain first the “L + ” dose of the toxin; this is the quantity which when mixed with one unit of standard antitoxin is just sufficient to kill a 250 grams guinea-pig in four days. Varying dilutions of the new antitoxin are then mixed with the L + dose and injected into guinea-pigs. Thus, the neutralising power of the new antitoxin can be compared quantitatively with the

standard and the number of units in a given volume stated. Antitoxin may also be titrated by the intracutaneous injections of toxin and antitoxin into the skin of the guinea-pig or rabbit, and, as a preliminary to the *in vivo* tests, by the flocculation reaction in mixtures of toxin and antitoxin; flocculation occurs quickest at the neutrality point.

The antitoxic property of serum is contained in the globulin fraction, and by precipitation with half-saturated ammonium sulphate the serum can be concentrated and much of the protein material which gives rise to serum sickness is removed. The Therapeutic Substances Regulations of 1927 and 1931 define the strength, quality and testing of diphtheria antitoxin, and these regulations must be observed by manufacturers supplying serum for therapeutic use.

Schick Reaction.—When a minute quantity of diphtheria toxin is injected intradermally, a local reaction follows if there is less than 1/100 of a unit of antitoxin present per c.c. of blood. This amount is sufficient under ordinary conditions to protect against an attack of diphtheria. The Schick test consists in the intradermal injection in the forearm of 1/50 of the minimum lethal dose (M.L.D.) of diphtheria toxin for a guinea-pig, diluted to 0.2 c.c. with a special buffer solution,¹ containing also 0.1 per. cent. Witte peptone. An old toxin preparation is chosen in which some of the toxin has changed to toxoid, so that 1/1000 unit of antitoxin should just neutralise the test dose. As a control, an equal amount of a similar dilution of the same toxin previously heated to 85° C. for ten minutes is injected intradermally into the other forearm. A positive Schick reaction consists in an area of redness and swelling appearing after twenty-four to forty-eight hours, reaching its maximum about the fourth day, when it measures 1.2 cm. or more in diameter. It persists for seven to fifteen days, and

¹ A mixture of 57 grams crystal borax, 8.4 grams boric acid, 99 grams sodium chloride is made; 1.5 grams of this mixture are dissolved in 100 c.c. of distilled water.

on fading shows superficial scaling and a persistent brownish pigmentation. The absence of reaction on either arm (*i.e.* "a negative Schick reaction") indicates that the toxin has been neutralised, sufficient antitoxin being present in the blood of the individual. A "pseudo-reaction" may occur, evidenced by an area of redness appearing early (within six to twelve hours) which is less intense and usually disappears in one to three days. If this appears in a Schick-negative person both forearms show similar reactions; in a positive reactor the unheated toxin produces a reaction which is more pronounced and more persistent than that due to the heated material. A convenient time to examine results is after seven days, when true reactions are still visible and most pseudo-reactions have faded. It is usually possible to determine without difficulty whether the result is positive or negative.

Under the Therapeutic Substances Regulations the reagents in the Schick test are designated "Schick Toxin" and "Schick Control," and the methods of manufacture must be in accordance with these regulations.

For test purposes a quantity of toxin representing one M.L.D., which is just neutralised by 1/20 unit of antitoxin, is obtained and diluted with 10 c.c. sterile buffer solution. The test dose—1/50 of the minimum lethal dose—is contained in 0.2 c.c.

The diluted toxin should be used as soon after being made as possible, but if prepared with the special buffer solution, and kept in the cold, it will remain fully active for many weeks.

Diphtheria Prophylactic.—Individuals having little or no antitoxin in the blood stream, as evidenced by a positive Schick reaction, may be actively immunised by the injection of one of the following preparations :

(1) Toxoid (F.T.): diphtheria toxin modified in toxicity by formalin and heat (*vide* p. 285). (2) Toxoid-antitoxin mixture (T.A.M.) in which the toxoid is partially neutralised by antitoxin. Three injections of 1 c.c. are made intramuscularly into the upper

arm, at intervals of two to four weeks. F.T. is a more powerful antigen than T.A.M., but is more likely to cause reactions after injection. It is therefore unwise to give an initial dose of 1 c.c. of this prophylactic to older children or adults unless a special test of sensitiveness to toxoid (the intradermal injection of diluted toxoid) has given a negative response. (3) Toxoid-antitoxin floccules (T.A.F.): a suspension of the precipitate of floccules formed when toxoid and antitoxin are mixed in appropriate neutralising amounts. Its immunising efficiency is high, and its tendency to cause reactions is slight. Unfortunately it is more costly to prepare than other prophylactics. The testing of all diphtheria prophylactics must be in accordance with the Therapeutic Substances Regulations.

Whatever prophylactic has been used in the past, it has never been safe to assume immunity unless a subsequent Schick test has given a negative result. With the more potent prophylactics which have recently become available it may shortly be possible to dispense with the Schick test for routine purposes. Thus, two injections of potent formol-toxoid, with or without the addition of a small amount of alum, or aluminium hydroxide (Al.F.T.), give extremely high immunity, but with somewhat more severe reactions than are encountered with T.A.M. or T.A.F.

For further details of the preparation of toxin, toxoid and antitoxin one of the larger works should be consulted.

DIAGNOSIS

A specimen of the throat secretion should be obtained. No antiseptics (*e.g.* in form of gargles, etc.) must have been applied within twelve hours. A sterile throat swab¹ should be rubbed over the affected area,

¹ A stiff wire with a pledget of cotton-wool firmly wrapped round one end for about $\frac{3}{4}$ in. The wire is kept in a narrow hard-glass test-tube, and for convenience the other end may be fastened to the cork which stoppers the tube. The tube with the contained wire and swab is sterilised by dry heat.

or, where there is no definitely localised lesion, over the mucous membrane of the pharynx and tonsils. A tube of Löffler's serum is inoculated by smearing the infected swab over the whole surface of the medium, moistening the swab in the condensation water at the foot of the tube. The tube is incubated for eighteen to twenty-four hours at 37° C.

The resulting growth is mixed by emulsifying it with a wire loop in the condensation fluid, and from this, films are made and stained either by (1) Löffler's methylene blue, (2) Neisser's method, or (3) Pugh's or Albert's stain (*vide* p. 161). Albert's stain can be specially recommended.

Films may also be made directly from the swab and stained by the above methods, and in a proportion of cases positive results may be obtained immediately. Cultures should, however, be made as a routine procedure, irrespective of direct examination.

In the case of suspected throat diphtheria, the appearance in cultures of bacilli showing the characteristic morphology and staining reactions (especially the metachromatic granules by Neisser's or Albert's stain) may be regarded as diagnostic.

In the case of supposed nasal diphtheria, diphtheria carriers, diphtheria affecting mucous surfaces other than the throat, and wound-diphtheria, the microscopic examination of cultures is not conclusive. The suspected organism must be isolated in pure culture and its virulence determined as described on p. 286.

It must be emphasised here that the responsibility for the diagnosis of diphtheria rests entirely with the clinician. The bacteriologist can merely state by the ordinary routine method the presence or absence of organisms morphologically resembling the bacillus in the specimen submitted to him. Failure to find such organisms does not necessarily exclude diphtheria, neither does their presence prove the disease to be

diphtheria.¹ If the clinician considers a case to be diphtheria it is his duty to administer antitoxin at once, and continue to do so even if a negative laboratory report is received. The mortality from the disease increases with the delay in administering antitoxin, and where there is reasonable suspicion that the case may be diphtheria, antitoxin must immediately be used without waiting for a bacteriological report. It is also emphasised that a reliable laboratory report, particularly in cases where there is doubt clinically, cannot be made under eighteen to twenty-four hours, and at that stage the bacteriologist can report on morphological appearances only. To prove conclusively the identity and virulence of the organism necessitates tests extending over several days, which are hardly practicable in ordinary routine diagnostic work. In order that the bacteriological report should be as helpful as possible, the utmost care must be taken that a suitable specimen is submitted in accordance with the directions given above and precise details as to the nature and source of the material should be furnished.

Allowance being made for the possible limitations of the routine diagnostic method described, the results of such examination have undoubtedly proved of the greatest value as an aid to, and confirmation of, the clinical diagnosis.

BACTERIA BIOLOGICALLY ALLIED TO THE DIPHTHERIA BACILLUS

BACILLUS OF HOFMANN (*Corynebacterium hofmanni*)

A throat commensal.

Morphology and Staining.—Compared with *B. diphtheriae* it is shorter (about 2μ) and may present a somewhat oval appearance; stained with Löffler's methylene blue, an unstained bar in the middle of the organism is a frequent character and renders it not

¹ See *A System of Bacteriology*, Medical Research Council, London, 1930, vol. v., p. 100.

unlike a diplococcus. It is strongly Gram-positive; usually no metachromatic granules are detected by Neisser's method.

Culture.—Grows aerobically on ordinary media; growths are more abundant than those of *B. diphtheriae*, and the colonies are larger and more opaque. For appearances on tellurite medium see p. 114.

Biochemical Reactions.—*Vide* Table (p. 294).

It is non-pathogenic to laboratory animals.

BACILLUS XEROSIS (*Corynebacterium xerosis*)

A commensal in the conjunctival sac. Closely resembles *B. diphtheriae*, and may show metachromatic granules.

Can be differentiated from *B. diphtheriae* by its production of acid in saccharose (*vide* Table, p. 294) and by its non-pathogenicity to laboratory animals.

ACNE BACILLUS (*Corynebacterium acnes*)

An organism associated with acne and regarded as the aetiological agent. It is Gram-positive, rod-shaped, and measures about 1.5μ by 0.5μ . It is markedly pleomorphic, and frequently shows a beaded appearance resembling a diphtheroid bacillus.

Culture.—In primary culture grows under anaerobic conditions or as a micro-aerophile if a fermentable carbohydrate such as glucose is present, or aerobically if the medium contains serum or blood and is definitely acid (pH 6.2–6.8).

OTHER DIPHTHEROID BACILLI

Certain of these present a close similarity to *B. diphtheriae*, and may exhibit the characteristic granules by Neisser's staining method (*vide* p. 282). They are mostly non-pathogenic, and have been isolated from the secretions of the nose and naso-pharynx, the ear, conjunctival sac, the skin, lymph glands (apart from disease) and other tissues, pus, wounds, etc. Compared with the true diphtheria bacillus they are of low virulence to laboratory animals.

BIOCHEMICAL REACTIONS OF *B. DIPHTHERIAE* AND CERTAIN ALLIED TYPES

	Glucose	Saccharose	Dextrin
<i>B. diphtheriae</i> . . .	±	-	±
<i>B. of Hofmann</i> . . .	-	-	-
<i>B. ærosis</i> . . .	±	±	-

(± - acid production)

BACILLUS PSEUDO-TUBERCULOSIS OVIS (PREISZ- NOCARD BACILLUS) (*Corynebacterium ovis*)

The causative organism of caseous lymphadenitis and pseudo-tuberculosis in sheep. A similar organism is associated also with ulcerative lymphangitis of horses (*vide infra*).

This organism is allied to *B. diphtheriae* in its biological characters.

Morphology.—Non-motile, slender rod-shaped organism 1–3 μ in length by 0.4 μ in breadth. When stained, it shows beading or a barred appearance like other diphtheroid bacilli, and is Gram-positive. By Neisser's method metachromatic granules can be demonstrated. Club-shaped forms may be noted.

Culture.—Growth occurs at 37° C. under aerobic conditions on ordinary nutrient media :

On agar, growth is at first scanty ; the colonies are small, thin and greyish-white in colour, folded and granular and often show concentric rings.

In gelatin no liquefaction occurs.

On Löffler's serum the colonies are similar to those on agar but exhibit a yellow colour.

In broth a granular growth occurs with sometimes a surface pellicle.

Glucose, maltose and dextrin are fermented, but not saccharose, lactose, mannite or glycerol. A haemolysin is produced in culture medium.

This organism produces an exo-toxin resembling to some extent that of the diphtheria bacillus. Guinea-pigs are highly susceptible to this toxin and show at the site of subcutaneous inoculation an inflammatory lesion with œdema and hæmorrhage, while the internal organs are congested and often contain small hæmorrhages ; there is however no change in the suprarenals and no pleural effusion (*cp. diphtheria*).

Occurrence.—The associated disease in sheep is chronic and characterised by involvement of lymphatic glands, which are enlarged and caseous. Caseous nodules are seen also in the

internal organs—*e.g.* lungs, spleen, liver and kidneys. The organism can be demonstrated in films or sections prepared from the various lesions.

Animal Inoculation.—Laboratory animals—*e.g.* guinea-pig—are susceptible to experimental infection with cultures. Intravenous injection produces a lethal effect within about ten days and at autopsy caseous areas are noted in internal organs—*e.g.* lungs and liver. Intraperitoneal injection in a male guinea-pig leads to involvement of the tunica vaginalis as in the case of experimental glanders (*vide* p. 311). Subcutaneous injection is followed by lymphatic gland involvement, the glands showing the characteristic caseation. Sheep and goats are also susceptible to experimental inoculation.

Diagnosis.—Films are prepared from the lesions and stained by Gram's method, methylene blue, and by Ziehl-Neelsen's method (to exclude acid-fast bacilli). Cultures are made, and pure growths from single colonies are investigated as regards cultural characters and experimental pathogenesis.

Similar organisms are found in equine ulcerative lymphangitis (pseudo-farcy), and in pseudo-tuberculosis (caseous lymphadenitis) of bovines ("*C. bovis*"). These organisms form a group of closely related types. They have frequently been classified as one species—the "*Preisz-Nocard Bacillus*." A similar organism (*B. pseudo-tuberculosis murium*) produces a disease in mice.

BACILLUS PYOGENES (*Corynebacterium pyogenes*)

An organism associated with suppurative lesions in pigs, cattle and certain other animals. It may occur in mastitis of cattle.

Morphology and Staining Reactions.—Non-motile, rod-shaped organism not usually exceeding 2μ in length and relatively slender. Shows great pleomorphism. Gram-positive in young cultures. Stained with methylene blue, diphtheroid forms may be seen with deeply stained bands or granules, but metachromatic granules are not usually observed in preparations stained by Neisser's method.

Culture.—Aerobe. Optimum temperature about 37°C . Grows best in media containing blood or serum, and in milk. The colonies on serum media are at first minute, but after several days' growth may attain a size of 2–3 mm. in diameter. They present no specially characteristic appearances. Growth on coagulated serum produces small pits of liquefaction. Gelatin is also liquefied. This organism is haemolytic when growing on blood-agar. In milk, acid and clot result in three days and after a time the clot is digested. Glucose, lactose and, in some cases, saccharose are fermented.

Pathogenesis.—In the natural infection in swine, suppurative lesions may occur in various parts of the body, liver abscesses and arthritis being specially frequent. In cattle, the organism has been found associated with a variety of suppurative lesions—*e.g.* abscesses, pyaemia, pyelitis, mastitis, etc.

Rabbits can be infected experimentally; intravenous injection of cultures produces a pyaemic condition with bone and joint lesions. Guinea-pigs are less susceptible.

CHAPTER XII

THE TUBERCLE BACILLUS AND OTHER ACID-FAST BACILLI

BACILLUS TUBERCULOSIS (*Mycobacterium tuberculosis*)

THE causative organism of tuberculosis in man, mammals and birds.

Under this designation are included different types—the “human,” “bovine” and “avian” types, so called in virtue of their occurrence in man, cattle and birds respectively.

HUMAN TYPE

Morphology.—Slender, straight or slightly curved rod-shaped organism, $2.5-3.5\mu$ by 0.3μ , with rounded, pointed, or sometimes expanded ends. In the tissues it may occur singly, or in pairs often forming an obtuse angle, or in small bundles of parallel bacilli. The bacillus is non-motile, and non-sporing though it possesses considerable powers of resistance to drying. In old cultures individual cells may grow out into long filaments and show branching.

Staining.—It is more difficult to stain than other bacteria. A strong dye with a mordant is required (*e.g.* carbol fuchsin—*vide* p. 158), and either prolonged staining or the application of heat. It may stain uniformly or show marked beading. When stained it resists decolorisation with 20–25 per cent. sulphuric or nitric acid, and also with alcohol, and is therefore described as “acid- and alcohol-fast.” The tubercle bacillus is Gram-positive, but can be demonstrated only with difficulty by Gram’s method. The “acid-fastness” is due to a lipoid substance in the

organism, which can be dissolved out with hot alcohol or ether.

It seems likely that the tubercle bacillus may occur in the tissues in a non-acid-fast form which is not demonstrable by the Ziehl-Neelsen method. It was originally claimed by Much that a granular phase of the organism could be recognised, and recently it has been stated that a filterable form of the organism can be demonstrated. This still requires fuller confirmation.

Culture.—Aerobe; temperature range—30°–41° C., optimum—37°–38° C.

Will not grow on ordinary media. Primary growths may be obtained on serum media, on pieces of animal tissue, or on a medium containing egg yolk (*vide* p. 109). In secondary culture, growths may result on ordinary media (agar, bouillon, potato) with 5–6 per cent. glycerol added; growth is slow—*i.e.* ten days may elapse after primary inoculation, or even sub-inoculation, before growth is apparent.

The most convenient medium for artificial culture in ordinary laboratory work is one of the glycerol-egg media (*vide* pp. 109–111); the growth is luxuriant and presents the following appearance:—dry, irregular, tough and tenacious, wrinkled or mammillated, at first white, later buff-coloured.

In glycerol broth—growth consists of white flocculi forming a powdery deposit, but if a fragment of inoculum is floated on the surface, growth may spread on the surface of the medium as a white wrinkled pellicle.

Viability.—The thermal death-point is about 60° C. While the majority of individual bacilli die when desiccated, a minority may survive for long periods. The organism is relatively resistant to injurious chemical substances: it can survive in putrefying material, it withstands the gastric juice, and in sputum resists 5 per cent. phenol, and antiformin (*vide* p. 302) for several hours. It is highly susceptible to sunlight and ultra-violet radiation. Cultures remain viable usually for several months.

Animal Inoculation.—The guinea-pig is susceptible to experimental infection. If injected subcutaneously

with the bacilli either in pathological material or in culture, after a few days a local swelling results consisting of tubercle nodules, which become confluent, undergo caseation and finally ulcerate. The neighbouring lymph glands become involved by spread of the bacilli along lymphatic channels and, later, lymph glands in other parts of the body are affected, showing the characteristic tuberculous lesions. The animal begins to lose weight, and dies in six weeks to three months. At autopsy, a general tuberculosis is noted; the spleen is enlarged and contains greyish-white tuberculous nodules or larger necrotic lesions. The liver and lungs, and also various other organs, present a similar condition.

If the animal is killed four or five weeks after injection, tuberculous nodules may be present only in the spleen and on the peritoneum.

Animals can be infected also by inhalation and by feeding.

Occurrence in Animal Tuberculosis.—The human type of tubercle bacillus, apart from its occurrence in human disease, has been found also in tuberculosis of monkeys, pigs and dogs.

BOVINE TYPE

Morphology and Staining reactions are practically identical with those of the human type.

Culture.—As compared with the human type, growth is less luxuriant, and the bovine type is described as “dysgonic.” On egg medium—it forms a thin, white, smooth, slightly moist, granular and easily broken-up growth (*cp.* human type—“eugonic”). The difference between the human and bovine types is accentuated by using a glycerol-egg medium. Glycerol favours the growth of the human type but has no influence on the bovine variety.

Pathogenicity to Animals.—The bovine type is more virulent to bovines and laboratory animals than the human type. In the ox it produces a fatal tuberculosis, whereas the human type causes only a localised lesion, which heals spontaneously.

The difference between the two types can be elicited by injecting a rabbit intravenously with an emulsion of 0·01–0·1 mgm. of dried bacilli (from a culture) emulsified in saline. The bovine type produces an acute generalised tuberculosis, and the animals usually die within two months; in the case of the human type the animals survive, or die only after two months, with slight lesions confined usually to lungs and (or) kidney.

The differentiation may also be brought out by injecting 10 mgm. of culture subcutaneously in the rabbit; the bovine type leads to a general tuberculosis, which is fatal usually within ten weeks, whereas the human type produces only a local lesion.

It is to be noted that strains which deviate in their characters from the standard human and bovine types may be met with. Thus, strains isolated from lupus are frequently of attenuated virulence for laboratory animals (Griffith).

The bovine type of tubercle bacillus, in addition to its association with tuberculosis of cattle, is the commonest variety found in tuberculosis of other domesticated animals (*e.g.* sheep, pigs, horses, cats).

OCCURRENCE OF TUBERCLE BACILLI IN HUMAN LESIONS

Both the human and bovine types are met with, though the former is the more prevalent. The latter is infrequently found in adults, but a proportion of tuberculous conditions in young subjects is due to this type, particularly cases resulting from alimentary infection by milk from tuberculous cows.

The percentage frequency of the bovine type of tubercle bacillus, according to Griffith, is as follows: tuberculosis of cervical glands, 85·7 in England and 84·9 in Scotland in children under five years, and 45·7 in England and 73·6 in Scotland at all ages; bones and joints, 18 in England and 42·8 in Scotland; genito-urinary system, 17·4 (England); lungs, 0·8 in England and 3·8 in Scotland; meningitis, 30·1 (England); lupus, 48·6 in England and 53·8 in Scotland.

Tubercle bacilli are most numerous in acute lesions showing rapid cascation—*e.g.* acute phthisis. In acute miliary tuberculosis they are relatively scanty. In chronic infections few tubercle bacilli are present, and they may not be detectable microscopically though demonstrable by animal inoculation—*e.g.* in the pus from a tuberculous abscess. In the lesions they are usually found free from cells, but intracellular bacilli may be noted.

In phthisis, tubercle bacilli are present in the sputum, and often in large numbers if the pulmonary lesion is active and rapidly breaking down.

In tuberculosis of the urinary system the bacilli may be found in the urine by microscopic examination of the deposit after centrifugalisation, but as a general rule they are relatively scanty, and may not be observed in film preparations.

In intestinal tuberculosis the bacilli may in some cases be found in films from the faeces.

In tuberculous meningitis, tubercle bacilli may be seen in films from the spinal fluid after centrifugalisation, but in early cases microscopic examination may yield negative results.

DIAGNOSIS

In general, microscopic examination is sufficient for ordinary diagnostic work.

Sputum—a film is prepared from the purulent portion of the sputum and stained by the Ziehl-Neelsen method (*vide* p. 158). A prolonged examination may be necessary in some cases where the bacilli are relatively scanty, and one examination with a negative result by no means excludes tuberculosis.

Urine, pleural, peritoneal and cerebro-spinal fluids—are centrifugalised, films are made from the deposit and stained by the Ziehl-Neelsen method. In the case of specimens of urine not taken with a catheter, it is essential to treat the film with alcohol (two minutes) after decolorisation with acid in the Ziehl-Neelsen process, in order to exclude smegma bacilli (*vide* p. 159).

Pus and faeces—films are made in the usual way.

In the case of tissues—sections are stained by the Ziehl-Neelsen method (*vide* p. 160).

Antiformin method for the detection of scanty tubercle bacilli in sputum, pus, tissues, etc.—

By this method the bacilli can be concentrated in the material examined, and it is particularly valuable where the bacilli are scanty. The method is of course entirely supplementary to the ordinary examination, and is quite unnecessary where bacilli can be seen in direct film.

“Antiformin” consists of equal parts of liquor sodae chlorinatae (B.P.) and 15 per cent. caustic soda. It has the property of dissolving cells and other bacteria, leaving acid-fast organisms intact.

A quantity of sputum or other material is treated with three or four times its bulk of antiformin diluted 1 in 6 with water, and the mixture is shaken and allowed to stand at 37°C. till it becomes thoroughly liquefied. This usually takes about an hour, but it is sometimes necessary to add more diluted antiformin to complete the solution. The mixture is then centrifugalised and the supernatant fluid is removed. Water is added, and mixed with the sediment. After centrifugalisation again, thick smears are made from the sediment, dried, fixed and stained by the Ziehl-Neelsen method.

Cultivation of Tubercle Bacilli from Pathological Material.—If the tubercle bacilli are likely to be present in pure culture in the material, tubes of Dorset's egg medium can be inoculated directly. If other organisms are present—*e.g.* in sputum—the antiformin method can be used and cultures made from the sediment; in this case the centrifuge tubes, water used for washing the sediment, etc., must be sterile.

Petroff's method (modified) is recommended for general routine use. Sputum is mixed thoroughly with three to four times its volume of 4 per cent. caustic soda, and placed in the incubator at 37°C. for

thirty minutes, the container being shaken from time to time. The mixture is centrifuged in a conical tube at 3000 r.p.m. for thirty minutes and the supernatant fluid poured off. The deposit is neutralised with 8 per cent. hydrochloric acid, which is added drop by drop, the reaction of the mixture being tested by touching litmus paper with a small loopful. The deposit is then inoculated on egg medium containing 1:10,000 crystal violet (Petroff's medium), which inhibits the growth of other organisms, or preferably on the Löwenstein-Jensen medium (p. 110). Pus may be treated by the same method, or with 6 per cent. sulphuric acid for the same time. In the latter case neutralisation of the centrifuged deposit with caustic soda should be carried out before the culture medium is inoculated.

A slower but more certain method of obtaining pure cultures is to inoculate a guinea-pig with the material, and to make cultures from the lesions in internal organs (*e.g.* spleen) after generalised tuberculosis results.

In laboratory diagnosis, where tubercle bacilli cannot be detected in specimens by microscopic examination, the *animal inoculation test* (guinea-pig) may be resorted to, and often yields positive results when microscopic examination is negative.

Tuberculin is a preparation consisting of the broken-up protoplasm of the bacillus *plus* its products in artificial culture medium. It was originally prepared from a six-weeks-old culture in glycerol bouillon, evaporated to one-tenth of its volume, sterilised by heat and filtered (Koch's "Old Tuberculin"). Various methods have been employed, however, in its preparation. Thus, Koch's "New Tuberculin" is derived by grinding the bacilli (obtained from a growth on solid medium) in 50 per cent. glycerol.

Tuberculin has been used in the diagnosis and treatment of tuberculosis and for full details one of the larger works should be consulted. The diagnostic application depends on the fact that the tissues of a person or animal infected with tuberculosis may exhibit hypersensitiveness (allergy) to the products of the bacillus—*e.g.* to tuberculin. Thus the subcutaneous injection of tuberculin in a tubercular subject may lead to local, focal and general reactions. Cutaneous allergy can be

elicited by the *cutaneous reaction of von Pirquet*, in which the "Old Tuberculin" is applied to a small abraded area of skin, or by the *intracutaneous test of Mantoux*, which consists in the intradermal injection of tuberculin (0.1 c.c. of a 1 : 1000 dilution) by means of a syringe with a fine needle. In both cases in a tubercular subject an area of erythema and swelling appears at the site of inoculation within a few hours and attains a maximum in twenty-four to forty-eight hours. It is to be noted, however, that such reactions do not necessarily denote active tuberculosis. The tuberculin reactions have been utilised also in the recognition of tuberculosis in cattle, and are of great importance in testing milch cows. Thus "certified milk" and "Grade A milk" must be obtained from tuberculin-tested animals which yield a negative reaction. The test in cattle is most reliably carried out by the *double intradermal method*: 0.1 c.c. Old Tuberculin is injected intradermally in a shaved area of skin in the neck, and the thickness of a fold of skin in this area is measured with callipers before the injection. The result is observed after forty-eight hours and a positive reaction is indicated by the occurrence of a hot, tender, diffuse and ill-defined swelling, the increased thickness of the fold of skin being 7-17 mm.; in non-tuberculous animals there may be some degree of infiltration, which is usually well defined and small in area; if the result after the first injection seems negative or inconclusive, a second dose is given at the same site and the result noted after twenty-four hours. A positive reaction is indicated by the same type of inflammatory swelling as that described above, the increased thickness of the skin fold being 13-40 mm.

By the Regulations under the Therapeutic Substances Act, the term tuberculin is restricted and applies to "preparations of fluid media in which *Bacillus tuberculosis* has been grown in artificial culture and which have been freed by filtration from the bacilli." These regulations stipulate that Old Tuberculin preparations shall be tested by an approved method for specific toxicity to guinea-pigs or other animals infected with the tubercle bacillus. A standard preparation of Old Tuberculin is kept in the National Institute for Medical Research, London, and Old Tuberculin shall not be issued if its activity differs from the standard preparation to such an extent that the difference is revealed by the test.

The *complement-fixation reaction* has been extensively applied both in diagnosis and in gauging the activity of lesions. The technique has been described in Chapter VII. The reaction is most frequent and

most marked in the case of chronic but active lesions. It is negative in many arrested cases, and it is noted that the reaction may be negative also in rapidly progressive tubercular conditions.

AVIAN TUBERCLE BACILLUS.—The causative organism of a tubercle-like disease in birds. Its morphology and staining reactions are the same as in the case of the other types of tubercle bacilli. Its optimum temperature is 40° C., and on glycerol-agar the growth is more rapid in development, moister, more homogenous and more luxuriant than that of the mammalian types. Individual colonies are large, raised, hemispherical, with a smooth shiny surface and a yellow or brownish-yellow colour.

It is highly virulent to fowls, which are resistant to the mammalian tubercle bacilli. For testing purposes 0.001 mgm. is injected intravenously or the bacilli are administered by feeding.

This type of tubercle bacillus may also occur naturally in pigs, and has occasionally been reported in human tuberculosis.

Acid-Fast Bacilli found in Cold-Blooded Animals.—Acid-fast bacilli resembling the tubercle bacillus have been isolated from fish, frogs, turtles, etc., and have been regarded as aetiologically associated with a tubercle-like disease in such animals. These organisms grow at low temperatures, even 15° C. In cultural characters they correspond to the avian type (*vide supra*).

Acid-Fast Saprophytic Bacilli.—Non-pathogenic acid-fast bacilli may be found in milk, butter, manure, and grass. They are similar in morphology to the tubercle bacillus, but their growth on culture medium is rapid; they develop on ordinary media and at room temperature, producing an abundant dry or slightly moist growth which is irregular, coarsely granular and sometimes wrinkled; most strains are definitely pigmented—yellow, pink or brown.

Smegma Bacillus.—This is a commensal organism found in the smegma and on the skin. It conforms in

biological characters to the saprophytic types described above. As it may occur in specimens of urine, it has to be differentiated carefully from the tubercle bacillus. It is generally shorter and thicker than the latter, and shows greater variation in size and shape. The smegma bacillus is acid-fast, but in urinary deposits is usually decolorised by alcohol (*vide* p. 159), which has no effect on the tubercle bacillus.

BACILLUS LEPRÆ (*Mycobacterium leprae*)

The causative organism of leprosy.

Morphology and Staining.—A straight or slightly curved slender bacillus, about the same size as the tubercle bacillus, with pointed, rounded, or club-shaped ends; so far as is known it is non-motile and non-sporing. Like the tubercle bacillus it requires as a rule a strong stain, and is acid-fast, though not to the same degree; it may stain uniformly, but usually shows marked beading, which is somewhat coarser than that of the tubercle bacillus; it is Gram-positive, and can be stained fairly readily by the ordinary Gram's method.

Culture.—A great many attempts have been made by various workers to cultivate this organism; the majority have been unsuccessful, and though successful results have been claimed and cultures of acid-fast bacilli have apparently been isolated from leprosy lesions, it is doubtful whether these strains represent the true leprosy bacillus. The attempted cultivation of this organism is hardly within the scope of routine practical bacteriology, and for further information on this subject the text-books and other literature should be consulted.

Occurrence and Distribution.—Leprosy is an infective granuloma, developing as (1) the "nodular" type, in which nodules of granulation tissue form in the skin, mucous membranes and various organs (*e.g.* lungs, liver, spleen, testes), or (2) the "maculo-anæsthetic" type, where the granulation tissue infiltrates certain nerves and leads to motor and sensory paralysis with

characteristic trophic changes (e.g. anaesthetic skin areas—"maculae"). Both types of the disease may occur in the same patient.

The organisms are found in the granulomatous lesions, being particularly numerous in the nodular form. They are distributed intracellularly for the most part, parallel bacilli occurring in bundles which may completely fill up cells. They may be found also in the tissue spaces, in the walls of small vessels, in skin glands, lymph glands, and in the secretions of the nose, throat and mouth, due to the fact that the mucosal lesions ulcerate readily and discharge bacilli into the mucous secretions. The organisms do not occur in the maculae which are essentially trophic and not primary leprous lesions. In leprosy the bacilli have actually been observed in organs without associated lesions.

The bacilli are present in the nerve granulomata but are less numerous than in the nodular lesions.

DIAGNOSIS

Films are made from any ulcerated nodule on the skin, or a non-ulcerated nodule can be punctured with a needle and squeezed till lymph exudes, from which films are made. Films can be prepared also from a scraping of an excised piece of tissue, or sections may be prepared as for histological examination. A convenient method is to remove with curved scissors a piece of skin (about 2 mm. deep) overlying a nodule and prepare films from the deep surface.

The films or sections are stained by the Ziehl-Neelsen method, substituting 5 per cent. sulphuric acid for 20 per cent. The presence of the characteristic acid-fast bacilli, especially when they occur in large numbers and are situated inside cells, is diagnostic.

As a routine measure, films should be made in all cases from the nasal mucosa or secretion, as diagnostic information may be obtained in this way even when nodules are not present in the skin. This also applies to the maculo-anaesthetic cases.

When the lungs are affected the bacilli may be demonstrated in the sputum, but require to be differentiated from the tubercle bacillus by animal inoculation; the leprosy bacilli do not produce any pathogenic effects in laboratory animals.

Bacillus (or *Mycobacterium*) *leprae murium*.—The organism of “rat leprosy,” which presents some pathological similarity to human leprosy, is an acid-fast bacillus related to, but not identical with, *B. leprae*. This disease of rats is transmissible experimentally to animals of the same but not other species. It should be noted that human leprosy cannot be transmitted to rats.

BACILLUS OF JOHNE'S DISEASE (*Mycobacterium paratuberculosis*)

The causative organism of a chronic enteritis of cattle, and a similar disease of sheep.

Morphology.—A Gram-positive, acid-fast and alcohol-fast bacillus like the tubercle bacillus, but more readily stained by the Ziehl-Neelsen method. It is comparatively short (1 to 2μ) and stains uniformly, though the longer forms may stain irregularly.

Culture.—Has proved exceedingly difficult to cultivate artificially. Twort and others have obtained growths on culture medium (e.g. egg medium) containing killed *B. tuberculosis* and other acid-fast bacilli—e.g. *B. phlei*—or extracts of these organisms. Cultures tend to resemble those of the tubercle bacilli.

Experimental Inoculation.—The disease is transmissible experimentally to calves, the incubation period being several months. Laboratory animals are insusceptible.

Distribution.—The lesions are of a granulomatous nature and lead to corrugated thickening of the mucosa of the intestine; the small bowel is primarily affected. The bacilli are present in large numbers, usually packed inside the cells of the lesion (as in leprosy).

Diagnosis.—At autopsy, the characteristic acid-fast bacilli may be demonstrated in the mucous membrane of the bowel by the appropriate staining methods.

During life, the organism may be observed in the excreta, especially after treatment with antiformin (*vide* p. 302). A diagnostic method which has been used in veterinary practice is to remove pieces of rectal mucous membrane with a Volkman's spoon, and from these to prepare films, which are stained by the Ziehl-Neelsen method. Tuberculosis may be

excluded by the inoculation of material containing the acid-fast bacilli into laboratory animals.

An allergic skin reaction evoked by "Johnin," a preparation (from cultures) analogous to tuberculin, has been utilized in diagnosis.

CHAPTER XIII

BACILLUS MALLEI; B. ANTHRACIS AND BIOLOGICALLY ALLIED ORGANISMS

BACILLUS MALLEI (*Pfeifferella mallei*)

THE causative organism of glanders.

Morphology.—Straight or slightly curved bacilli with rounded ends, about $3-5\mu$ by 0.4μ . Shorter forms are frequently noted and also longer filaments. Bacilli with club-shaped ends, and even branched forms have been observed. In old cultures swollen irregular involution forms are numerous. The bacilli occur singly or in pairs. They are non-motile and non-sporing.

Staining.—Gram-negative. An important feature of the organism, as seen in the tissues and inflammatory exudate, is its granular or beaded appearance.

Culture.—Aerobe; optimum temperature— $35^{\circ}-38^{\circ}\text{C}$.; does not grow below 25°C .; grows on ordinary media, but the addition of glycerol (4 per cent.) assists growth.

Agar stroke—uniform, white, semi-transparent, moist band of growth along needle track, which later becomes opaque, somewhat slimy and yellowish-brown in colour. This growth is not specially characteristic.

Potato slope—at first a transparent yellowish growth (“honey-like”), later becoming opaque and of a chocolate-brown colour.

Colony variants presenting a dry and wrinkled appearance have been observed.

The only carbohydrate fermented is glucose, but the reaction is slight and is not uniformly obtained.

In primary culture, growth does not occur readily and the organisms die out quickly, but after several subcultures they become adapted to a saprophytic existence, and may live for two months.

Occurrence.—Glanders is an infective granuloma, with a marked tendency, however, to suppurative change. It is essentially a disease of horses, asses and mules, and is only occasionally transmitted to man, usually by direct infection from an animal source.

In acute and subacute animal glanders, ulcerating nodules occur in the nasal mucosa and later in the lungs and internal organs. The bacilli are present in considerable numbers in all the lesions, situated for the most part extracellularly.

In chronic animal glanders ("farcy"), where the superficial lymph glands and vessels are involved, the bacilli are less numerous.

Latent infections are not infrequent in animals, and have also been observed in the human subject.

In human glanders, the infection usually originates in the skin (*e.g.* wound, abrasion, etc.), more rarely in the mucosa of the mouth or nose. The bacilli are found in the local inflammatory lesion and spread by the lymphatics, producing an acute lymphangitis. Ultimately a pyaemic condition results with secondary foci, in which the bacilli are numerous.

Animal Inoculation.—In equidae, the typical disease can be reproduced by subcutaneous injection of recently isolated cultures; asses are most susceptible. Guinea-pigs are markedly susceptible, and after subcutaneous injection die in a week or two with generalised lesions, as in acute animal glanders. If a male guinea-pig is inoculated intraperitoneally, the tunica vaginalis is rapidly invaded, and externally swelling of the testis is noted (Straus reaction). Even when pathological material containing *B. mallei* in mixed infection is injected intraperitoneally, the specific organism seems to flourish best in the tunica, and can be isolated more easily from this situation than from the peritoneum.

DIAGNOSIS

Films are prepared from the pus, discharge from sores, etc., or from nodules in internal organs found at post-mortem; these are stained with methylene blue and by Gram's method. The appearance of beaded Gram-negative organisms corresponding to *B. mallei* is suggestive. It should be noted that glanders bacilli are not easily demonstrable in preparations from the lesions.

Cultures are also made on glycerol-agar, and if a mixed growth results, pure cultures are obtained from single colonies. The chocolate-coloured growth on potato is an important criterion in identification.

In all cases the nature of the infection must be confirmed by animal inoculation. A male guinea-pig is injected intraperitoneally with the pathological material or the culture isolated; in two or three days an enlargement of the testis results, and the animal subsequently dies, showing the lesions of acute glanders (*vide supra*). If pyogenic organisms are also present in the material injected, and a septic peritonitis results, the glanders bacilli will be found more numerous in the tunica vaginalis. If the inoculum contains a large number of other organisms it may be introduced by subcutaneous injection.

Agglutination Test.—In the diagnosis of glanders in horses, the agglutination test has been used. The technique followed is that described on pp. 196–199, with certain minor differences. The bacterial suspension is prepared from young glycerol-agar slope cultures which, before emulsification, are placed at 60° C. for two hours to kill the organisms. For personal safety it is necessary to use dead cultures. Serum dilutions ranging from 1 in 100 to 1 in 4000 are tested. The agglutination tubes are incubated for twenty-four hours at 37° C. before readings are made. Normal horse serum may agglutinate the bacillus in low dilutions, and only agglutination in high titres, 1 in 1000 or more, can be accepted as proof of infection. Agglutination in

lower titres does not, of course, exclude the existence of the infection. Any spontaneous agglutination in the control would, of course, invalidate the result; some workers include parallel tests with normal horse serum as an additional control.

A *complement-fixation* test (*vide* p. 35) has also been employed as a serum diagnostic method. It has been regarded as more reliable than the agglutination test.

Mallein is a preparation analogous to tuberculin. A four-weeks' culture in glycerol bouillon is sterilised at 100° C., and then filtered through a porcelain filter. The filtrate contains the heat-stable soluble products of the organism and constitutes the preparation, mallein, which is utilised for diagnostic purposes in a manner analogous to tuberculin. Subcutaneous injection of 1 c.c. diluted mallein (containing 0·1 c.c. of the crude preparation) in an infected animal produces a local reaction (a swelling reaching a diameter of five inches after twenty-four hours) and a general reaction manifested by a definite elevation of temperature. A conjunctival reaction may be elicited readily by introducing mallein into the conjunctival sac, and is a more satisfactory test than that in which the mallein is injected subcutaneously.

BACILLUS WHITMORI (*Pfeifferella whitmori*)

The causative organism of Melioidosis—a glanders-like disease occurring in the Malay States where it is epizootic among rodents and from these animals transmissible to man. The disease has been reported also in Cochin China and Ceylon. The organism is similar to *B. mallei*, but is motile and grows in gelatin at 20° C., liquefying the medium. Mucoid and corrugated types of growth on agar have been described and a brown growth on potato similar to that of *B. mallei*. Glucose, lactose, saccharose and mannite are fermented (without gas production). Susceptible animals (*e.g.* guinea-pig, rat) may be infected experimentally. In the male guinea-pig the Straus reaction occurs as in animals infected with *B. mallei*. *B. mallei* and *B. whitmori* are serologically related.

BACILLUS ANTHRACIS

The causative organism of anthrax in animals and man.

Morphology.—A non-motile, straight, rod-shaped, sporing bacterium, rectangular in shape and of

relatively large size— $4-8\mu$ by $1-1.5\mu$. The bacilli tend to be arranged in chains end to end (streptobacillus), but may occur singly and in pairs; in blood and tissue they exhibit a distinct capsule when suitably stained. The spore appears first as a refractile dot in the centre of the bacillus, increasing in size till it can be seen as an oval structure, central in position and of the same cross-diameter as that of the bacillus. Sporulation occurs readily when the organism is discharged from the body of an infected animal, and spores are a morphological feature of the bacilli when growing in artificial culture, but *sporulation does not occur in the tissues*. After the spore is fully formed the residual protoplasm of the bacillus disintegrates and the spore is left as a free structure. The spore represents a highly resistant phase of the organism, and can survive under conditions which would be unfavourable to the vegetative form. When replaced in favourable conditions, the spore capsule ruptures at one pole and the vegetative phase is reproduced from the contained protoplasm.

Staining.—Gram-positive. The spore is unstained by the ordinary methods, but can be stained differentially by special methods (*vide* p. 162).

Methylene-Blue Reaction of McFadyean.—This staining reaction has been utilised in veterinary work for the recognition of anthrax bacilli in blood films. The films are made in the usual way on slides, dried and passed rapidly three times through the flame; they are then stained with polychrome methylene blue (*vide* p. 151), washed and dried. Between the bacteria an amorphous purplish material is noted, representing the disintegrated capsules of the organisms; this appearance is characteristic of the anthrax bacillus.

Culture.—Acrobe and facultative anaerobe; temperature range— $12^{\circ}-45^{\circ}$ C., optimum— 35° C.; grows on all ordinary media; aerobic conditions are necessary for sporulation, for which the optimum temperature is $25^{\circ}-30^{\circ}$ C.

Colonies on agar—white, granular, circular discs

(about 3 mm. in diameter) which, under the low power of the microscope, show a wavy margin, often likened to locks of hair. The colony is one continuous convoluted thread of bacilli in chain formation.

Agar stroke—thick, white, opaque, somewhat dry, friable growth with notched edges, showing the same microscopic characters as the colonies. To the naked eye this growth presents a “ground-glass” appearance.

Gelatin stab—a line of growth along needle puncture, from which fine lateral spikes radiate—longest towards the top. This is the so-called “inverted fir-tree growth”; later liquefaction occurs, starting at the top of the growth.

Coagulated serum is partially liquefied.

In bouillon—growth develops as white flakes which sediment, and sometimes with pellicle formation.

Growing on blood-agar the anthrax bacillus is only slightly haemolytic as compared with the “anthracoid” bacilli (*vide* p. 320) which are markedly lytic.

Glucose, saccharose and maltose are fermented (without gas production).

Variation.—Capsule formation is subject to variation, and when the capsule is absent or imperfectly developed the colonies tend to be moist and slimy and may be devoid of the characteristic wreathed margins. This is well seen in cultures which have been attenuated in virulence by growth at temperatures above the optimum (*e.g.* 42°–43° C.) as in Pasteur’s method of attenuating the organism for prophylactic vaccination.

The typical colony, as described, is of the “rough” form; the variant is small, “smooth” and without the characteristic wreathed appearance, while the bacilli in this type of colony are arranged in bundles, not in a convoluted chain. Virulence is associated with the “rough” form, the “smooth” variant being relatively avirulent.

Viability.—The thermal death-point of the vegetative form is about 60° C. The spore can withstand 100° C. (moist heat) for five to ten minutes, and resists desiccation for an indefinite period.

Occurrence in Animal Lesions.—The anthrax bacillus produces an epizootic disease in herbivorous animals, particularly among sheep and cattle. The condition is usually septicaemic in nature, and, post-mortem, the

bacilli are found in large numbers in the heart, blood and internal organs, especially in the *spleen*, which is enlarged, soft and diffuent.

Occurrence in Human Lesions.—Transmission of the infection to man is from an animal source:

- (1) Infection may occur through the skin—*e.g.* in persons handling infected animals, carcasses or hides, from shaving brushes, etc.—the resulting lesion being described as a “malignant pustule”—*i.e.* an area of intense inflammation, with a central slough and with surrounding inflammatory oedema. Lymphatic spread may occur, and even septicæmia.
- (2) Infection may result from inhalation of spores carried in dust or filaments of wool from infected animals, as in the wool factories—“wool-sorters’ disease.” The organisms settle in the lower part of the trachea or in a large bronchus, and an intense inflammatory lesion results, with hæmorrhage, oedema, spread to the thoracic glands, involvement of the lungs, and effusion into the pericardial and pleural cavities; the organisms are present in considerable numbers in the lesions; a septicæmic condition may also supervene.
- (3) Infection may occur by the intestine, but this is relatively uncommon in man.

Infection in Animals.—Animals may be infected from pasture which has become contaminated with *B. anthracis* spores derived from previous cases of the disease in the particular area. The disease is also spread by artificial foodstuffs—*e.g.* oil-cake. Biting flies—*e.g.* *Stomoxys*—may convey the organism, though in an entirely mechanical way.

Experimental Inoculation.—Guinea-pigs and white mice are most susceptible. If a guinea-pig is injected subcutaneously with pathological material containing the bacilli, or with cultures, the animal dies usually within two days, showing a marked inflammatory lesion at the site of inoculation and extensive gel-

atinous oedema in the subcutaneous tissues. Large numbers of bacilli are present in the local lesion. The animal shows a profound septicaemia, and the anthrax bacilli are present in large numbers in the heart blood and in the capillaries of internal organs. They are *especially numerous in the spleen*, which is enlarged and soft.

Prophylactic Immunisation.—Pasteur's vaccine has been extensively applied in the prevention of the disease among animals. The vaccine is a culture of the bacillus attenuated by growing at 42°–43° C. The degree of attenuation is, however, difficult to regulate. Besredka has advocated the intradermal inoculation of the vaccine (as opposed to the subcutaneous injection of the older method) and claims that a solid immunity can be produced in this way. A "combined" method of prophylactic immunisation has also been applied—*viz.* injection of Pasteur's vaccine along with an immune serum.

Therapeutic Antiserum.—The serum of artificially immunised animals—*e.g.* Selavo's serum—is used in the treatment of human anthrax, and is able to confer passive immunity.

DIAGNOSIS

Malignant Pustule—

1. Films are made from the exudate and stained by Gram's method; the finding of bacilli morphologically like *B. anthracis* is suggestive but not conclusive.

2. Successive stroke inoculations should be made on an agar plate. The resulting colonies are recognised by examining them with the low power of the microscope, and films are made and stained by Gram's method. Spores are noted in cultures.

3. In all cases the identity of the suspected organism must be confirmed by inoculation of a guinea-pig or mouse with exudate from the lesion, or with the isolated culture. A small dose of culture is sufficient to produce a lethal effect. The occurrence of the bacilli in the heart blood and in the spleen in considerable numbers, and the other post-mortem appearances described above, are diagnostic.

Diagnosis of Anthrax in Animals (post-mortem).—The usual form of post-mortem examination must not

be made—*i.e.* to prevent any distribution of sporing bacilli from the carcase. In the body no sporulation occurs, but spores are readily formed when the bacilli are exposed to air. A film of blood taken from a superficial vein in the ear is prepared, and stained by Gram's method and by McFadyean's methylene-blue method (*vide supra*). The finding of characteristic bacilli in the blood giving the methylene-blue reaction is practically diagnostic. In pigs and horses the bacilli may not be detectable in the blood. If necessary, the organism can be cultured and identified by the procedure described above, using a specimen of blood from the ear for the investigation.

Examination of Shaving Brushes, Wool, etc., for
B. anthracis

The bristles or wool are cut up with scissors into small fragments and shaken thoroughly with several volumes of sterile salt solution so as to obtain "washings" from the material. The saline is decanted and centrifugalised. The sediment is suspended in about 1 c.c. of saline and heated at 80° C. for half-an-hour to eliminate any non-sporing organisms present. Cultures on agar plates are then made from the suspended sediment and guinea-pigs are injected subcutaneously with the material, and with cultures of any suspicious organism isolated if the direct inoculation test is negative. It is to be noted that in such examinations, organisms very similar in their morphological and cultural characters to the anthrax bacillus may be encountered—e.g. "*B. anthracoides*" (*vide infra*). Such organisms, if injected in large doses, may also produce fatal effects in mice and guinea-pigs. Their differentiation from *B. anthracis* is referred to later.

Precipitin Test.—This test was first used by Ascoli in the recognition of anthrax infection in organs and tissues from suspected carcases, and may be applicable even in the case of putrefied material. It depends on the occurrence of a specific precipitin (*vide p. 35*) in the serum of an artificially immunised animal. Immune sera, however, vary in their precipitin content, and for the test a serum with known precipitating properties must be selected. About 2 grams of the tissue are boiled for five minutes with 5 c.c. of normal saline, to which acetic acid has been added in the proportion of 1 : 1000. The fluid is cooled and then filtered through asbestos. 0.5 c.c. of the serum is

placed in a narrow tube and the filtrate is carefully run on to the top. The development of a white ring of precipitate at the junction of the two fluids within fifteen minutes denotes a positive result.

THE AEROBIC GRAM-POSITIVE SPORING BACILLI BIOLOGICALLY ALLIED TO *B. ANTHRACIS*

These organisms are saprophytes and represent a large number of different species. They are found in soil, water, dust and air. Being ubiquitous, they are frequent contaminants of culture medium in the laboratory, and bacteriological workers should be acquainted with their general biological characters.

Classical types representative of this group are *B. subtilis* (the "Hay bacillus"), *B. mycoides*, *B. mesentericus* (*vulgatus*), *B. megatherium*, "*B. anthracoides*." The type species is *B. subtilis*, and for convenience the group is often spoken of as the "*B. subtilis* group." For the detailed differential features of the various species, reference can be made to *Bergey's Manual of Determinative Bacteriology* (3rd edition). The general characters of the commoner types met with in laboratory work may be summarised as follows, and for general purposes it is unnecessary to identify a particular species :—

Morphology.—Certain types tend to resemble the anthrax bacillus and young forms are Gram-positive. Some are morphologically identical with *B. anthracis* and occur in similar chain formation. Others are shorter with rounded ends, and several motile species with peritrichate flagella are met with (e.g. *B. subtilis*). The spore is central or eccentric (e.g. *B. mycoides*, *B. subtilis*), subterminal or terminal. The bacillus is not usually altered in shape by sporulation, though in some cases there may be some swelling of the rod when the spore is fully formed (e.g. *B. subtilis*).

Culture.—The optimum temperature is usually low—e.g. 18°–22° C.—but certain types grow best at 35°–37° C. and some are thermophilic; they are characteristic aerobes but usually also facultative

anaerobes; abundant growth occurs on all the ordinary culture media. The appearances of growths vary considerably among different types. *B. subtilis* produces a white, glistening, adherent, somewhat membranous growth, which tends to spread, and somewhat similar growths are seen among other species. Certain types produce colonies and growths practically similar to *B. anthracis*, with the same "wreathed" appearance of the margins—e.g. "*B. anthracoides*." The colonies of *B. mycoides* are at first similar to those of the anthrax bacillus, but are easily differentiated by their spiky or feathery appearance due to projecting and branching threads radiating out from the central growth. The growths may be dry, gummy or moist, and white, greyish-white, yellowish or brown. Certain species producing a black pigment have been described. On potato, characteristic cultural appearances may be noted—e.g. *B. mesentericus* develops a thick wrinkled or folded layer of growth which assumes a brownish colour. Pellicle formation on bouillon is a frequent character. Generally, gelatin is liquefied: in some cases the liquefaction is stratiform (e.g. *B. subtilis*) but different forms of liquefaction are noted in other species. Some types ferment carbohydrates. Starch may be hydrolysed.

These organisms are usually non-pathogenic on experimental inoculation into laboratory animals.

"*B. anthracoides*."—This designation has been applied to a type of organism of the *B. subtilis* group which in morphological and cultural characters closely resembles *B. anthracis* and may exhibit colonies with the "wreathed" margins characteristic of the latter. Under certain conditions this organism might at first be confused with the anthrax bacillus. It can be differentiated usually, however, by its motility. It is to be noted that if a large dose of culture of this type of organism is injected into a guinea-pig or white mouse, a local inflammatory lesion with inflammatory oedema may be produced and septicaemia with a

lethal effect. The organism can be detected in the heart blood and internal organs, though *in small numbers* (*cp.* anthrax). In blood or tissues it does not exhibit the McFadyean methylene-blue reaction (*vide* p. 314).

CHAPTER XIV

THE GRAM-NEGATIVE AEROBIC BACILLI OCCURRING AS COMMENSALS OR PATHOGENS IN THE INTESTINE, AND ALLIED BACTERIA; ENTAMOEBA HISTOLYTICA AND OTHER INTESTINAL PROTOZOA; ACIDURIC INTESTINAL BACILLI

THE GRAM-NEGATIVE INTESTINAL BACILLI

THIS class includes the following main groups :—

1. *B. coli* group.
2. Typhoid-paratyphoid or “ enteric ” group.¹
3. Food-poisoning group.
4. Dysentery group.

General Characters.—Gram - negative, non-sporing bacilli, about 2–4 μ by 0.5 μ (average)—aerobes and facultative anaerobes—growing best about 37° C.—not liquefying gelatin or solidified serum.

The various groups and species are differentiated by cultural, biochemical and serological tests.

BACILLUS COLI GROUP (*Escherichia*)

This group includes a considerable number of different types, generally designated by the generic

¹ The basis of this classification is clinical rather than biological—e.g. the food-poisoning organisms belong biologically to the paratyphoid group, which is frequently spoken of as the *Salmonella* group.

term *B. coli*, occurring normally in the large intestine of man and other mammals.

Morphology and Staining.—Gram-negative bacilli, 2–4 μ by 0.5 μ , but filamentous forms up to 8–10 μ in length may occur, and short cocco-bacillary forms are not infrequent. The different types vary in motility. The motile varieties show a peritrichate arrangement of their flagella.

Culture.—Aerobe and facultative anaerobe. Temperature range—10°–44° C., optimum—37° C. Grows abundantly on ordinary media.

Colonies on agar—relatively large, thick, greyish-white, moist, circular discs; the opacity and size vary with different strains; some strains produce colonies and growth of a viscid or mucoid consistence; the margin of the colonies may be circular or wavy. “Rough” colony variants may be observed in some strains.

Gelatin stab—a white line of growth develops along the needle track, with a disc at the top on the surface of the medium; gas spaces form in the gelatin due to the fermentation of the natural muscle sugar of the meat infusion which is the basis of the medium; no liquefaction occurs.

In bouillon—a uniform turbidity.

Potato slope—white moist growth, later assuming a brownish colour.

Biochemical Reactions.—The most prevalent types of *B. coli* (“typical *B. coli*”) in the intestine exhibit the following reactions:—

Motility	Glucose	Lactose	Dulcitol	Saccharose	Alonitol	Inulin	Inositol	Indole	Voges and Proskauer reaction
±	+	+	±	±	—	—	—	+	—
								(<i>vide</i> <i>infra</i>)	(<i>vide</i> <i>infra</i>)

(*Vide* p. 96 for method of testing, and Table on p. 358*a*)

The *B. coli communis* (Escherich)—*Escherichia communis*—one of the commonest of these types, has the following characters:—

+	+	+	+	—	—	—	—	+	—
---	---	---	---	---	---	---	---	---	---

+ (under various carbohydrates) signifies acid and gas formation.

Indole Production.—This is tested for by growing the organism in peptone water, and after two days withdrawing with a sterile pipette 2 or 3 c.c. into a test-tube. An equal volume of Ehrlich's rosindole reagent is then added :

Para-dimethyl-amido-benzaldehyde	.	.	4 grams
Absolute alcohol	.	.	380 c.c.
Pure hydrochloric acid	.	.	80 c.c.

A rose colour develops in the presence of indole, and can be separated out with amyl alcohol. The addition of a saturated solution of potassium persulphate hastens the reaction. If the indole reaction is negative after two days' growth, the test should also be repeated after ten days, as some strains are slow in their production of indole.

Voges and Proskauer Reaction.—This reaction is exhibited by certain atypical varieties of *B. coli*. A strong solution of caustic potash is added to a two-days' glucose-bouillon culture, and the culture is then allowed to stand at room temperature for a few hours. If the reaction is positive an eosin-like colour develops, due to a reaction between diacetyl (formed by oxidation of acetyl-methyl-carbinol) and the peptone or one of its constituents.

Methyl-red Reaction (*vide* p. 235).

Citrate-utilisation Reaction (*vide* p. 236).

Other Biochemical Reactions common to the typical *B. coli*.—On MacConkey's bile-salt neutral-red lactose agar—the colonies are rose-coloured, due to the action on the neutral red of the acid produced from the lactose.

In litmus milk—acid and clot produced.

In media containing neutral red—a green fluorescence develops, especially under anaerobic conditions.

In media containing nitrates—nitrates reduced to nitrites.

Carbon dioxide and hydrogen are produced by the fermentation of glucose in the following proportions: $\text{CO}_2 : \text{H}_2$ —1 : 1–2.

Some strains, particularly those isolated from urinary infections, are haemolytic towards human red cells. This property can be elicited best by growing the organism in a fluid medium to which blood has been added.

ATYPICAL VARIETIES OF *B. COLI*

- (1) Non-gas-producing types are occasionally met with—*B. coli anaerogenes* (*vide* Table, p. 358a).
- (2) Non-indole-formers (*vide* Table).
- (3) Certain non-lactose-fermenters. Some of these may ferment lactose after spontaneous mutation in culture (*vide* Table).
- (4) *B. aerogenes* subgroup ("inosite fermenters"). The so-called *B. lactis aerogenes* belongs to this subgroup. These organisms also ferment adonite, and some exhibit the Voges and Proskauer reaction. They are non-motile and are characterised by their large, raised, slimy or viscid colonies. The citrate-utilisation reaction is frequently positive, the organism growing in Koser's citrate solution (*vide* p. 236). The $\text{CO}_2 : \text{H}_2$ ratio (*vide supra*) may differ from that of the typical *B. coli*, being approximately 2 : 1.

B. cloacae.—This organism is generally regarded as closely related to the *B. coli* group, but differs in respect of its liquefaction of gelatin (*vide* Table on p. 358a). It has been found in sewage and polluted water.

The *B. coli* Test in Water Examination is referred to on p. 234.

Occurrence of B. coli Group.—Reference has already been made to their occurrence normally in the intestinal contents, where they are present in very large numbers. These organisms are also potential pathogens. They are found most frequently in pyogenic infections of the urinary tract (pyelitis, cystitis, etc.) either in pure culture or mixed with pyogenic cocci, cholecystitis and cholangitis, appendix abscess, peritonitis.

DIAGNOSIS

In film preparations from pus, urinary sediment, etc., stained by Gram's method, *B. coli* can be recognised as a small Gram-negative bacillus, but the morphological characters of this organism are not specific. If cultures are made on MacConkey's medium, their identity can be established at once *in the case of the typical forms*, by their rose-pink colonies.

It must be remembered, however, that certain strains of *B. coli*, even those which actively ferment lactose in fluid medium, may produce pale colonies on MacConkey's medium, so that for identification it may be necessary to isolate the particular strain, and test its reactions in detail.

BACILLUS OF FRIEDLÄNDER (PNEUMOBACILLUS)

(*Klebsiella pneumoniae*)

Originally described as a causative organism of pneumonia.

Morphology and Staining.—A small non-motile, non-sporing, Gram-negative bacillus with rounded ends and varying greatly in size—1–4 μ by 0.5–1 μ . The shorter forms simulate cocci. The bacilli occur usually in pairs, but also singly, and in short chains. They are typically capsulate, especially when seen in the tissues.

Culture.—In cultural characters it resembles the inosite-fermenting subgroup of *B. coli*—e.g. *B. lactis aerogenes*. The colonies exhibit a characteristic mucoid or viscid consistence, already referred to on p. 325.

Biochemical Reactions.—Different strains vary. Some correspond closely with the inosite fermenters of the *B. coli* group (*vide* Table, p. 358*a*). Others are non-lactose fermenters. Some strains form gas slowly, others apparently fail to produce gas in fermentation tests.

This organism may generally be regarded as biologically related to the *B. coli* group, though its

habitat as a commensal or pathogen is generally in the upper respiratory passages.

Occurrence in Human Disease.—Friedländer's bacillus is only rarely responsible for cases of lobar pneumonia. It is frequently associated, however, with catarrhal conditions of the respiratory tract, suppuration in nasal sinuses, meningitis, conjunctivitis, empyema, etc.

Serological Classification.—Three serological types have been defined (analogous to the types of pneumococci); a certain proportion of strains, however, cannot be identified with these types and form a serologically heterogeneous group analogous to Group IV of the pneumococcus. Type-specificity depends on a carbohydrate substance contained in the bacterial capsule. It is of special interest that the specific substance of one of these types can be identified serologically with the specific substance of Type II pneumococcus.

BACILLUS OF RHINOSCLEROMA (*Klebsiella rhinoscleromatis*).—This organism closely resembles Friedländer's bacillus in morphology and cultural characters, but produces no gas from glucose, and does not ferment lactose. It is associated with a chronic granuloma of the mucous membrane of the nose, mouth, or throat. The bacilli can be seen in the lesions situated intracellularly. The disease is prevalent only in South-Eastern Europe.

BACILLUS "OZAENAE" (*Klebsiella ozaenae*).—Closely resembles Friedländer's bacillus, but is non-gas-producing in glucose. It is found associated with ozaena, but its aetiological relationship is doubtful.

TYPHOID PARATYPHOID OR "ENTERIC" GROUP

This group includes:

B. typhosus.

B. paratyphosus A.

B. paratyphosus B.

Two organisms closely allied to *B. paratyphosus B* have also been noted in certain cases of enteric fever, and designated *B. paratyphosus C* (*vide* p. 337).

Note.—It is convenient to classify the typhoid and paratyphoid infections together as "enteric fever."

BACILLUS TYPHOSUS (*Eberthella typhi*)

The causative organism of typhoid fever.

Morphology and Staining.—A Gram-negative non-sporeing bacillus, about $2-4\mu$ by 0.5μ , actively motile, due to numerous long flagella (peritrichate); filamentous forms (8μ) are common.

Culture.—Aerobe and facultative anaerobe; optimum temperature— 37°C . Grows well on ordinary media.

Colonies on agar—like those of *B. coli*, but smaller, thinner and more transparent; cultures may show a mixture of “rough” and “smooth” colonies (*vide* p. 22).

Gelatin stab—like *B. coli*, the growth is less abundant, and without gas formation. No liquefaction occurs.

Colonies on MacConkey's medium—smaller than *B. coli* and colourless, *B. typhosus* being a non-lactose-fermenter.

Biochemical Reactions.—Ferments glucose and mannite with acid but no gas formation; does not ferment lactose or saccharose and does not produce indole. See Table on p. 358*a*.

Viability.—The thermal death-point is about 60°C . The majority of individual bacilli die within a few hours when subjected to drying. In water the bacilli gradually die, but may survive for some days.

Occurrence.—This organism shows a selection for lymphoid tissue—*e.g.* the Peyer's patches and lymphoid follicles of the intestine, the mesenteric glands, and spleen. Thus it attacks the Peyer's patches of the small intestine, and leads to an acute inflammatory reaction, and infiltration with mononuclear cells, necrosis, sloughing and the formation of the characteristic typhoid ulcers. It is present in large numbers in the inflamed tissue, in the ulcers, and is found in the intestinal contents and the dejecta. The mesenteric glands show similar inflammatory changes.

At an early stage of the disease—during the first week or ten days—and in relapses, a condition of bacteriaemia exists, and the typhoid bacillus can be demonstrated by blood culture. It is also present in the spleen, occurring in clumps. Typhoid bacilli are frequently present in large numbers in the gall bladder. They may localise in the kidney and appear in the urine, sometimes producing a marked bacilluria. Localisation may also occur in bone-marrow.

The bacillus is found in other lesions occurring as complications or sequelae of typhoid fever—*e.g.* acute suppurative osteitis, abscess of the kidney, acute cholecystitis, broncho-pneumonia, empyema, ulcerative endocarditis. Even in the suppurative lesions it may be present in pure culture.

Infection is by ingestion; from the small intestine the organism passes by the lymphatics to the mesenteric glands, and invades the blood possibly *via* the thoracic duct; the liver, gall bladder, spleen, kidney, bone-marrow, etc., then become infected from the circulation; from the gall bladder a further invasion of the intestine results and the bacilli may then appear in the faeces in appreciable numbers (*vide* p. 331).

In 2 to 5 per cent. of convalescents, the typhoid bacillus persists in the body sometimes for indefinite periods. In such “carriers” the bacilli are present in the gall bladder, or in foci in the kidney, and are excreted in the faeces or urine.

B. typhosus cannot exist as a saprophyte outside the body, but it can survive long enough to be transmitted by some vehicle of infection—*e.g.* sewage and polluted water, shell-fish, and vegetables contaminated with human excretal matter, house flies, articles of food contaminated by carriers, etc. Milk is a good culture medium for the typhoid bacillus, so that contamination of milk—*e.g.* by carriers engaged in dairy work—is a likely source of outbreaks of the disease.

Serological Reactions.—The serum of animals immunised with typhoid bacilli contains agglutinating antibodies (*vide* p. 30) which are specific for the

characteristic antigens of the organism, and an anti-serum is therefore employed in the identification of this species (*vide* p. 200).

The antigenic structure of this organism in relation to allied species is shown in the Table on p. 343.

BACILLUS PARATYPHOSUS A (*Salmonella paratyphi*)

BACILLUS PARATYPHOSUS B (*Salmonella schottmülleri*)

Causal organisms of paratyphoid fever, which is, for all practical purposes, clinically similar to typhoid.

The morphology and general cultural characters are identical with those of *B. typhosus*.

For their biochemical reactions see Table on p. 358a. The chief sugar reactions are: lactose—no change; glucose, mannite and dulcitol—acid and gas; saccharose—no change. It is to be noted that the paratyphoid bacilli can be distinguished biochemically from *B. typhosus*. Biochemical differences between the paratyphoid bacilli may also be elicited (see Table) but the ultimate identification depends on agglutination reactions.

Their *occurrence* and *distribution* in the body of an infected person are the same as in the case of the typhoid bacillus.

B. paratyphosus A is more common in the East, whereas *B. paratyphosus B* is the commoner in Europe. In Western Europe the paratyphoid bacilli have in the past produced a relatively small proportion of enteric cases, but in recent years in this country *B. paratyphosus B* infections have assumed a greater relative prevalence.

Serological Reactions.—These organisms possess different antigens, and agglutinating antisera for known strains are used for the identification of the respective species. The paratyphoid bacilli are similarly differentiated from *B. typhosus*.

The antigenic structure of these organisms in relation to other members of the *Salmonella* group is shown in the Table on p. 343.

DIAGNOSIS OF ENTERIC INFECTIONS

The bacteriological diagnosis depends on (1) the isolation from the body, and the identification of the causative organism, or (2) the demonstration of its presence in the body by the agglutination or Widal reaction, which is based on the occurrence of specific agglutinins to the organism in the serum of the infected person.

In the early stage, blood culture is the most conclusive diagnostic method, and should be employed, if possible, in all cases met with during the first seven to ten days of the illness, and in relapses (where a bacteriological diagnosis has not previously been established). The possibility of demonstrating typhoid - paratyphoid bacilli in the blood is much less at later stages. The method is referred to on p. 141. If the result is positive the strain is isolated in pure culture, and identified by morphological, cultural and biochemical characters (see Table, p. 358a), and by testing it with an agglutinating antiserum to a known *B. typhosus*, *B. paratyphosus A*, or *B. paratyphosus B*, according to the biochemical reactions observed. Agglutination should occur approximately to the end-titre of the serum (for the strain used for immunisation).

The typhoid and paratyphoid bacilli may also be isolated from faeces and urine.

FAECES.—*B. typhosus* is most frequent in the third week, *B. paratyphosus A* about the twelfth day, and *B. paratyphosus B* at the end of the second week or the beginning of the third week. Examination of faeces may frequently yield negative results unless repeated, and the isolation of these organisms from faeces is often rendered difficult owing to their being relatively scanty as compared with *B. coli*.

In submitting specimens of faeces for culture, if there is likely to be a delay of some hours before the laboratory cultivation can be carried out, 2 volumes of 30 per cent. neutral glycerol in 0.6 per cent. sodium chloride should be added to

1 volume of the faeces and thoroughly mixed with it. This obviates the overgrowth of the typhoid-paratyphoid bacilli by the other intestinal organisms present.

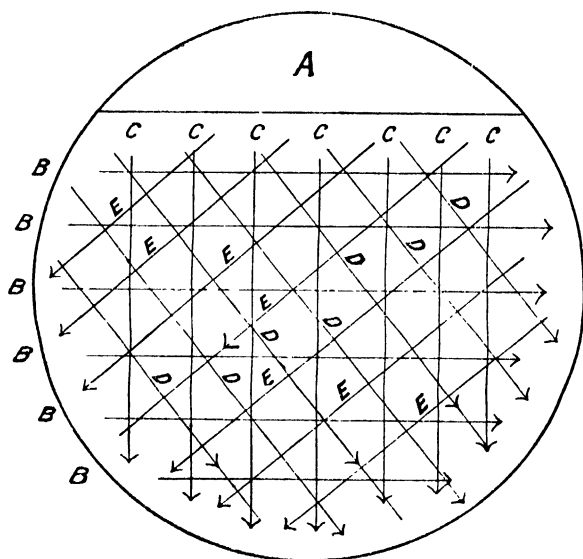
Direct plating of Faeces.—The *medium* recommended is MacConkey's bile-salt neutral-red lactose agar (*vide* p. 111). It is simple in composition, easily prepared and stable as compared with other differential media for intestinal organisms. On this medium *B. typhosus* colonies are "pale" as compared with the pink colonies of *B. coli*. MacConkey's medium is also sufficiently inhibitory to the common dust-borne organisms to facilitate manipulation of plates in drying and inoculation. *It is essential that the surface of the medium should be sufficiently dry before inoculation.* If even a small amount of condensation water is present on the medium, a confluent growth results instead of separate colonies.

The inoculation is made by successive strokes as follows:—

A loopful of the specimen (liquid faeces or a dense emulsion in saline from solid or semi-solid faeces) is smeared over area A of the plate (see diagram, p. 333). The loop is sterilised in the flame, re-charged by rubbing it over area A, and then used to inoculate the remainder of the plate by successive parallel strokes, B, C, D and E drawn in the directions indicated in the diagram. The wire should be held so that the whole loop is in contact with the surface of the medium. In this way the resulting colonies are evenly distributed over the plate.

The result depends on the concentration of organisms in the specimen, and on the size of plate used. Plates of 6 in. diameter should be employed if possible. It is essential that the plates be abundantly inoculated, but if the specimen contains an excess of organisms, it may be difficult to obtain satisfactory separation of the colonies on a small plate. The method described allows a heavy inoculation to be made with the resulting colonies well separated (except of course in area A). The plates are

incubated overnight. After eighteen to twenty-four hours the colonies are usually sufficiently large for "picking off" those that are considered likely to belong to the enteric group. Some workers incubate the plates for longer periods if no suspicious colonies are noted after twenty-four hours, and occasionally this procedure is of value. The suspicious colonies can



be recognised by their "pale" appearance, but other intestinal organisms may produce colourless colonies on MacConkey's medium—even strains of lactose-fermenting *B. coli* (*vide supra*). Several of these colonies are subcultured on agar slopes, and for this purpose a straight wire is used for subinoculating them from the plate.

The pure cultures isolated are tested and identified as indicated under blood culture (*vide supra*).

The Brilliant-green Enrichment Method for the Isolation of the Enteric Group from Faeces.—This method depends on the

selective inhibition of the prevalent types of *B. coli* by brilliant green. Thus in fluid media containing certain concentrations of the dye, the typhoid-paratyphoid bacilli grow well, while the growth of *B. coli* is partially or completely restrained, and the former organisms can be enriched at the expense of the latter. Occasionally, however, unusual types of coliform bacilli that are resistant to brilliant green may overgrow the enteric organisms, and direct plating (*vide supra*) should be carried out in parallel with the enrichment.

Method.—If the stool is solid or semi-solid, a dense emulsion is prepared in sterile saline solution, and from this, or the liquid stool, three tubes of 10 c.c. of peptone water (2 per cent. peptone, 0·5 per cent. sodium chloride, pH 7·0), containing the following amounts of a 1 : 10,000 watery solution of brilliant green, are inoculated :

1	2	3
0·25 c.c.	0·4 c.c.	0·7 c.c.

The 1 : 10,000 solution is made up when required from a stock 1 per cent. solution by the addition of 0·1 c.c. to 10 c.c. of sterile water, and the appropriate amounts are added to the peptone tubes.

In general a large loopful of liquid faeces or faecal emulsion is added to the tubes. If, however, the stool is watery, three or four loopfuls should be used for the inoculation of each tube. The tubes are incubated at 37° C., and, after twelve hours, subcultures on MacConkey plates are made from each.

Under favourable conditions practically pure growths of the enteric organisms may be obtained in one or more of the brilliant-green tubes. Even when pure growths do not result, the typhoid or paratyphoid bacilli are relatively numerous as compared with *B. coli*, so that their isolation is more easily accomplished.

This method yields better results with *B. paratyphosus* than with the typhoid bacillus.

Wilson and Blair's bismuth sulphite medium may also be used with advantage to facilitate the isolation of enteric group organisms (*vide p.* 113).

URINE.—The urine is centrifugalised, several loopfuls of the deposit are inoculated on a MacConkey plate, and successive strokes made in the usual way so that isolated colonies are obtained. In enteric fever there appear to be transient bacilluric periods, and repeated examinations of the urine are of particular

value where the isolation of the causative organism is aimed at, and where other methods have been unsuccessful.

WIDAL REACTION.—The technique is described in Chapter VII.

The reaction begins to appear *usually* about the seventh to the tenth day. Occasionally it is earlier in development (*e.g.* fifth day), but may be delayed. A negative result at an early stage of the illness therefore may be inconclusive. The Widal reaction, tested quantitatively, is also progressive up to a certain point—*i.e.* the end-titre of the reaction rises from the time of the first appearance of agglutinins in the serum. A “rising titre” is therefore highly significant in diagnosis.

As a rule, both O and H agglutinins are developed, but in some cases only the O agglutinin is demonstrable (probably due to the antigenic characters of the infecting strain); this must be borne in mind in carrying out the Widal test (*vide* p. 198).

Enteric carriers usually exhibit a positive reaction.

Persons inoculated with the typhoid-paratyphoid vaccine show specific agglutinins in their sera, and this complicates the interpretation of the Widal reaction in such persons. In previously vaccinated cases a definitely rising titre for any one of the enteric group has been regarded as significant from the diagnostic standpoint. It has been shown also that in such persons the agglutinins are mainly of the H type, whereas in infected subjects both O and H agglutinins can be demonstrated. By testing with alcoholised bacterial suspensions (*vide* p. 201) the presence of O agglutinins in the serum can be determined, and this method has been utilised for the Widal reaction in vaccinated persons.

It must be remembered that normal serum may also agglutinate the typhoid and paratyphoid bacilli in low dilutions, and no diagnostic significance can be attached to the Widal reaction unless marked

agglutination, by the method described, occurs in the following dilutions of serum :—

- B. typhosus* 1 in 60 or higher dilutions.
B. paratyphosus A 1 in 30 or higher dilutions.
B. paratyphosus B 1 in 120 or higher dilutions.

This applies to the average strains and cultures of the typhoid-paratyphoid group used for the Widal test. Different strains and different cultures of the same strain may vary to some extent in agglutinability, and attempts have therefore been made to standardise the reaction by using formolised bacterial suspension each batch of which has been compared in agglutinability with a given standard suspension. In this way a factor can be ascertained by which results with a particular batch can be made comparable with those obtainable if the standard were used (*vide* p. 200). The formolised suspensions are suitable for testing only the H agglutinin. Alcoholised suspensions, however, may be used in parallel with formolised preparations for testing the O agglutinin (*vide supra* and p. 201).

In ordinary routine work the method described and the criteria of a positive reaction indicated above serve well for carrying out the test and interpreting results.

If agglutination occurs only in low dilutions, the test should be repeated. Later results may show higher end-titres and are therefore more conclusive.

Routine Bacteriological Examination of suspected Enteric Cases.—If duration less than ten days, carry out blood culture, adding 5 c.c. blood to 50 or 100 c.c. of 0·5 per cent. sodium taurocholate solution (*vide* p. 142).

If blood culture negative, and when duration is more than ten days, draw blood for Widal reaction with *B. typhosus* and paratyphoid bacilli; if negative, repeat serum tests in three or four days, and submit specimens of faeces and urine for bacteriological examination; if no positive findings still available, repeat Widal test and examination of faeces and urine.

DIAGNOSIS OF TYPHOID AND PARATYPHOID CARRIERS

In a considerable proportion of carriers the Widal reaction is positive, and the test is of some value as a preliminary method, but a negative result is of no significance.

The proof that a person is a carrier depends on the isolation of *B. typhosus* or *B. paratyphosus* from the faeces or urine, and at least three successive examinations should be made before the result is declared negative.

As the bacilli are likely to be most numerous in the bile and in the contents of the small intestine, three grains of calomel followed by a saline purgative should be given, and after catharsis, the second or preferably the third stool is used for the examination. The specimen should be cultured as soon as possible after it is passed. The methods used are as described above. The brilliant-green enrichment method should be employed in parallel with direct plating and yields better results in the isolation of *B. paratyphosus*.

The urine is examined as in the diagnosis of enterica.

Typhoid-paratyphoid Vaccine

This is prepared from "smooth" cultures of *B. typhosus*, *B. paratyphosus A* and *B.*, according to the method described on p. 227. The vaccine is sterilised at 55° C. (twenty minutes). For prophylactic use two doses are usually given subcutaneously at intervals of seven to ten days.

	1st dose	2nd dose
<i>B. typhosus</i> . . .	500 million	1000 million
<i>B. para. A</i> . . .	375 "	750 "
<i>B. para. B</i> . . .	375 "	750 "

The prophylactic value of this vaccine has been fully established.

BACILLUS PARATYPHOSUS C

Two types of organisms have been designated *B. paratyphosus C*, both occurring in cases of enteric fever. One is the monophasic type of *B. suipestifer* (*vide infra* and p. 343); the other is the "Hirschfeld" type (*vide* Table on p. 358*a*), which is also related to this organism though possessing a different flagellar antigen in the specific phase (see Table on p. 343). Infections due to the latter have been reported mainly in Eastern countries; the former occurs in Western Europe.

A type of paratyphoid bacillus called the "*Sendai*" type has been noted in Japan. It resembles *B. paratyphosus A* (see Table on p. 343).

Certain non-lactose-fermenting types related to the *coli* and typhoid-paratyphoid groups have been described as causal organisms of enteric-like fevers in the East—e.g. *B. asiaticus*, *B. columbensis*.

ISOLATION OF BACILLUS TYPHOSUS AND PARATYPHOID BACILLI FROM WATER

Various methods have been used for the purpose, but it is difficult to isolate these organisms even from a water known to be polluted with infective excretal matter. The following method is recommended for the purpose—to 900 c.c. of water in a sterile stoppered flask 100 c.c. of a sterile 10 per cent. solution of peptone containing 5 per cent. sodium chloride are added, and then 5 c.c. of a 1:1000 solution of brilliant green in distilled water. The flask is incubated and sub-inoculations are made on MacConkey plates after twenty-four and forty-eight hours. The enrichment principle applied in this method is referred to on p. 333.

Wilson and Blair's medium (*vide* pp. 113, 334) has been used successfully in isolating *B. typhosus* from water.

THE FOOD-POISONING GROUP (*Salmonella*)¹

These organisms are associated with cases of "meat or food poisoning," in which the illness takes the form of an acute gastro-enteritis with marked toxæmia. The incubation period may be short, symptoms occurring even a few hours after the ingestion of the contaminated food. Preserved meats (e.g. sausage) are most frequently responsible. The food may not show any obvious evidence of bacterial contamination. The *Salmonella* organism growing in the food produces an active endotoxin which may be responsible for the early symptoms in cases in which the incubation period is short. This toxin is generally stable at 100° C. for thirty minutes.

Occurrence.—These organisms are found in the intestinal contents in the disease, and may produce also a bacteriaemia.

As regards the source of the infection: the animal

¹A detailed study of the Food-poisoning group has been reported by Savage and White; see *Medical Research Council's Special Report Series*, Nos. 91 and 103.

from which the flesh is derived may have been infected previous to death, or the meat may have been contaminated from some extraneous source. Organisms of this type occur in certain animals—*e.g.*, cattle, pigs. They produce epizootic infections in rats and other rodents. Human carriers have been noted, and these may be responsible for contamination of foods.

Organisms of the *Salmonella* group have also been found in the eggs of infected pigeons and ducks, and cases of food-poisoning in the human subject have occurred after the consumption of such infected eggs.

BACILLUS ENTERITIDIS OF GAERTNER (*Salmonella enteritidis*)

This organism is similar to *B. paratyphosus B* in its various general characters (*vide* Table, p. 358*a*), but can be differentiated by its agglutination reactions—*i.e.* an antiserum to a known strain of *B. enteritidis* agglutinates strains of the same species, but has no action on *B. paratyphosus B*. Similarly, *B. paratyphosus B* antiserum has no effect on *B. enteritidis* unless in low dilutions. These two organisms are clearly differentiated in their antigenic constituents. It may be noted here that the somatic antigen of *B. typhosus* is the same as that of *B. enteritidis* (see Table on p. 343).

This organism is responsible for infection in cattle and in rats. It is extremely virulent to laboratory animals by parenteral inoculation, and even by enteral administration of culture, producing a haemorrhagic enteritis and bacteraemia.

BACILLUS AERTRYCKE (*Salmonella aertrycke*)

Similar to *B. paratyphosus B* (*vide* Table, p. 358*a*). Its differentiation from *B. paratyphosus B* by serological reactions is complicated by the occurrence of marked co-agglutination of *B. paratyphosus B* by *B. aertrycke* antisera, and similarly *B. paratyphosus B* antisera agglutinate *B. aertrycke*. This depends on

the fact that these two organisms possess certain antigenic constituents in common, though each in the specific phase contains a different flagellar antigen (see Table on p. 343).

Absorption tests, however, enable differentiation to be established (*vide* p. 203). If a *B. aertrycke* serum (diluted) is mixed with a dense emulsion of *B. paratyphosus B*, and after interaction for three hours at 37° C. the bacteria are removed from the mixture by centrifugalisation, it is found that the co-agglutinins (for *B. paratyphosus B*) have been removed from the serum, while the specific agglutinins for *B. aertrycke* are unaffected. Similar absorption of the serum with *B. aertrycke* removes *both* the specific agglutinins and the co-agglutinins.

These effects may be summarised as follows:

	—Agglutination of—	
	<i>B. aertrycke</i>	<i>B. paratyphosus B</i>
Antiserum for <i>B. aertrycke</i>	+	+
After absorption with <i>B. paratyphosus B</i> .	+	-
After absorption with <i>B. aertrycke</i>	-	-

For purposes of diagnostic identification, an unknown strain should be able to absorb from a *B. aertrycke* antiserum the specific agglutinins for this organism.

The identification of *B. aertrycke* and other *Salmonella* types is dealt with in further detail later (p. 342).

B. aertrycke produces epizootic enteritis in guinea-pigs and mice ("mouse typhoid"). It is less frequently

found in rats. Infection occurs in pigs and in certain birds. Thus an organism of this type was originally described in psittacosis (*vide* p. 475); it is not related to this disease.

OTHER TYPES OF THE SALMONELLA GROUP FOUND IN FOOD POISONING

According to Savage and White, *B. aertrycke* is the commonest cause of food-poisoning in this country; *B. enteritidis* is second in prevalence. An organism designated the "Mutton type," which has been recorded frequently, is identical with *B. aertrycke*. Another type, designated "Newport," is not infrequent in occurrence. Rarer types are the so-called "Stanley" and "Derby" types, and still further varieties of the *Salmonella* group have been described (see Table on p. 343). These organisms exhibit the general biological characters of the group and are differentiated by their serological reactions, agglutinin-absorption tests being necessary to distinguish them from related types, including *B. paratyphosus B.*

B. suispestifer (*Salmonella suispestifer* - Bacillus of hog-cholera) was originally regarded as the causal agent of swine-fever or hog-cholera, which is now considered to be due to a filterable virus. *B. aertrycke* and *B. suispestifer* have been confused and organisms designated *B. suispestifer* have been recorded in cases of food poisoning. It would appear, however, that the true *B. suispestifer* occurs infrequently in this condition. The biochemical reactions of this organism are given in the Table on p. 358*a*, and reference to it has been made on p. 337 in relation to "*B. paratyphosus C.*" Identification is established by agglutination and agglutinin-absorption tests with an antiserum for a known strain (see Table on p. 343).

DIAGNOSIS

The stools are plated out directly on MacConkey's medium as in the diagnosis of enteric fever, pale colonies are picked off, and the resulting cultures are tested and identified. Detailed agglutination and absorption tests are necessary for identification of the *Salmonella* type present (*vide infra*).

Blood culture may in some cases yield positive results, and should be carried out as a routine measure.

In convalescence, the serum of patients agglutinates the homologous organism, but the agglutination test is

not applicable during the acute stage, as the agglutinins take some days to make their appearance in the blood.

Bacteriological examination of suspected articles of food, if available, should be carried out.

IDENTIFICATION OF TYPES OF THE SALMONELLA GROUP

When a non-lactose-fermenting bacillus presenting the general characters of this group has been isolated from a case, its precise identification is often a matter of considerable difficulty. The presence or absence of motility should be carefully noted and a complete set of sugar media inoculated (including xylose, arabinose, inosite and rhamnose) along with lead acetate agar. The biochemical reactions are of value when considered in association with the antigenic structure.

As will be seen from the Table opposite, the identification of the O antigen (*vide* p. 34) provides a convenient method of placing any member of the group into one of a number of subgroups. To assign the unknown bacillus to its subgroup, sera prepared by immunising animals with alcoholised suspensions of representative members of each subgroup are used in agglutination reactions with an alcohol-treated O suspension of the unknown. A convenient set of such O sera are those for *B. paratyphosus* B, *B. enteritidis* Gaertner, the Newport type, *B. suipestifer* (monophasic) and *B. paratyphosus* A. Small-flake agglutination to the end-titre of the serum would indicate the nature of the O antigen present. The results of the direct agglutination reaction may then be confirmed by an agglutinin-absorption test.

The next step is to determine whether the culture (if diphasic) is in the specific or group phase (*vide* p. 34). An H-agglutinating serum prepared against *B. suipestifer* (monophasic; European type) is a convenient reagent for this purpose, its H agglutinin being exclusively of "group" character. If a formolised suspension of the unknown bacillus is agglutinated in large floccules to any extent by this serum, it is in the group phase, and an effort must be made to secure a specific-phase subculture of it before further identification is attempted.

Plates are inoculated so as to yield discrete colonies, and a number are examined. Separate colonies are touched with an inoculating wire and some of the growth in each case emulsified in a drop of a low dilution of *suipestifer* (monophasic) serum on a slide. If the serum is known to give rapidly visible floccular agglutination with known group antigens by this method it is sufficiently reliable as a preliminary test. A colony, organisms

THE SALMONELLA GROUP AND SEROLOGICALLY ALLIED ORGANISMS¹

Arranged in Subgroups with common O antigens

Type.	O antigen	H antigen	
		Specific Phase	Group Phase
<i>paratyphosus B</i>	I. II.	Specific	G. A.
<i>aertrycke</i>	I. II.	"	G. A. B. C.
Stanley	I. II.	S.	G. A.
Reading	II.	D ₁ .D ₂ .	G. B. E ₁ . E ₂ .
Derby	II.	R. Q.	..
<i>abortus equi</i>	II.	D ₁ .N ₁ .N ₂ .	..
Binns	I. II.	..	G. A. B. C.
<i>enteritidis</i> Gaertner . . .	III.	R. P ₁ .P ₂ .	..
Dublin	III.	R. P ₁ .P ₃ .	..
<i>typhosus</i>	III.	S.	..
Dar-es-Salaam	III.	D ₁ .N ₁ .M ₁ .Y.	..
<i>pullorum</i>	III.	non-motile	..
<i>gallinarum</i>	III.	non-motile	..
Eastbourne	III.	D ₁ .D ₂ .	G. E ₁ .F ₂ .
Tokyo	III.	R. P ₁ .P ₂ .	G.
Newport	IV.	D ₁ .D ₂ .	G. A. B. C.
<i>morbificans bovis</i> . . .	IV.	Specific	G. E ₁ .E ₂ .
<i>suipestifer</i> (diphasic : American) .	V.	W.w ₁ .	G. E ₁ .F ₂ .
<i>suipestifer</i> (monophasic : European) .	V.	..	G. E ₁ .F ₂ .
<i>paratyphosus C</i> (Hirschfeld)	V.	W.w ₂ .	G. E ₁ .F ₂ .
<i>paratyphosus A</i>	VI.	T.	..
Sendai	VI.	T.	G. E ₁ .

¹ For further details of the serology of these organisms see Lovell, *Bull. of Hygiene*, 1932, vol. 7, p. 405; Smith, *J. Hyg.*, 1933, vol. 33, p. 224.

from which do not agglutinate with the group serum, is subcultured as probably in the specific phase and then retested macroscopically. (The group $\rightarrow$ specific dissociation of the H antigen may be hastened by repeated growth in broth containing any "group" serum diluted 1:10.)

When the specific phase has been obtained formalised suspensions are used in agglutination reactions with pure specific-phase H sera prepared against the *Salmonella* types in the subgroup to which the already determined O antigen belongs. Again agglutinin-absorption tests should be used to confirm the results of positive direct agglutination reactions observed.

In most cases the evidence thus obtained, in association with the biochemical reactions, will serve to identify the unknown organism with sufficient accuracy. In the case of diphasic types the group phase may be investigated in the same way by agglutination and agglutinin-absorption tests with known group-phase antisera. This step will, of course, be necessary in the case of monophasic types found in the group phase only — e.g. "Binns" and European (monophasic) *suispestifer*.

Where the bacillus does not appear to conform precisely to any known type, O, H (specific) and H (group) antisera must be prepared and reciprocal absorption tests carried out with antigens and sera of all strains with which it appears to be related.

It should be noted that where the bacillus under investigation has undergone variation to the "rough" form its O antigen can no longer be identified, while a non-motile variant can be allocated only to its appropriate subgroup, since it lacks the H (flagellar) antigens necessary for complete serological identification.

In the Table on p. 343 the numbers and letters designate the various antigenic components which have been identified. The term "specific" denotes that the H antigen is associated with that particular organism only and not with any other.

The Table serves to illustrate the extreme complexity of antigenic structure and yet close inter-relationship of the organisms of the *Salmonella* group, and also indicates the great difficulties, technical and otherwise, of the ultimate identification of an organism which has given rise to an outbreak of food poisoning.

BACILLUS PULLORUM (*Salmonella pullorum*)

The causative organism of "white diarrhoea" of chicks.

In general biological characters this organism shows some correspondence to the typhoid-paratyphoid group, but is non-motile. Its biochemical reactions are given in the Table (p. 358a);

gas formation is not abundant. The organism is present in the faeces, blood and internal organs—e.g. liver, spleen—and at autopsy can be isolated readily on nutrient agar. It has also been found in the ovaries of adult birds (which have become carriers), in egg yolks and in the yolk-sacs of developing chicks. The serum of infected birds agglutinates the bacillus, and this reaction has been utilised in controlling the spread of the disease among flocks. Agglutination by a serum dilution of 1 in 50 is diagnostic. An agglutinating anti-serum is employed in identifying strains. This organism shows a close serological relationship to *B. typhosus*, possessing the same somatic antigen (which is also common to *B. enteritidis*); agglutinin-absorption tests (*vide* p. 203) may be applied to confirm the results of direct agglutination tests (see Table on p. 343).

BACILLUS GALLINARUM (*Salmonella gallinarum*—*Bacillus* of Fowl Typhoid)

This organism, associated with a disease of fowls characterised by severe anaemia, resembles the typhoid-paratyphoid group in general characters, but is non-motile and does not produce gas from sugars. Its fermentative reactions are shown in the Table on p. 358*a*. It has the same somatic antigen as *B. typhosus* and *B. pullorum*, and is serologically indistinguishable from the latter (see Table on p. 343).

B. abortus equi (*Salmonella abortus equi*) is an organism of the *Salmonella* group associated with contagious abortion of mares. Its biochemical reactions are shown in the Table on p. 358*a*. Agglutinin-absorption tests with a specific antiserum are required for its identification (see Table on p. 343).

GROUP OF DYSENTERY BACILLI

The causative organisms of an acute form of dysentery most prevalent in tropical and subtropical countries, but occurring also in temperate climates—e.g. asylum dysentery, infantile diarrhoea.

Morphology and Staining.—Non-motile, non-sporing, Gram-negative bacilli about 2–4 μ by 0.5 μ , but showing a tendency to shorter cocco-bacillary forms. Filamentous forms are less common.

Culture.—In cultural characters resemble *B. typhosus*. Gelatin is not liquefied.

Biochemical Reactions.—They all ferment glucose (without gas production), and in the case of all sugar

fermentations are non-gas-producing. The dysentery group can be subdivided into types according to the fermentation of lactose, dulcitol, saccharose, mannitol and maltose, the production of indole from peptone and agglutination reactions with specific antisera. The classical types are *B. dysenteriae* Shiga (*Shigella dysenteriae*), *B. dysenteriae* Flexner, and *B. dysenteriae* Y (Hiss and Russell). The Flexner and Y types are so closely related that, for convenience, they may be classified together and designated *B. dysenteriae* Flexner-Y (*Shigella paradysenteriae*).

The biochemical reactions are shown in the Table on p. 358a.

The Shiga type is also identified by agglutination with an antiserum to a known Shiga strain, and is clearly differentiated in serological characters from the other types of dysentery bacilli.

Organisms with the biological characters of the Flexner-Y subgroup possess at least four different antigenic constituents, designated V, W, X and Z, one of which tends to predominate in any strain. Thus different serological types can be separated according to the predominant type of antigen. In certain strains, however, the different antigens are more or less evenly balanced—e.g. the classical Y strain; an antiserum for this strain has therefore been used in the serological identification of the whole subgroup.

Experimental Inoculation. Cultures of dysentery bacilli are generally non-pathogenic when introduced by the mouth in laboratory animals. Intravenous injection produces a haemorrhagic enteritis, and if the animal survives muscular paralysis may result. These effects are specially marked in the case of the Shiga type, which forms a potent exotoxin.

Besides the classical types, other dysentery bacilli are met with which do not react to a Shiga or Y antiserum, and yet have the general characters of the dysentery group (as stated above). These are often designated *atypical dysentery bacilli*, and their biochemical characters are indicated in the Table. A

number of types have been recognised differing according to their biochemical characters.

Sonne type.—This organism conforms to the characters of the subgroup of atypical dysentery bacilli, and has been observed as a causal agent of dysentery in Norway, Sweden, Denmark and in this country. It represents a distinct serological type. In biochemical reactions it at first resembles the Flexner type, but produces late fermentation of saccharose and frequently lactose. Indole production is, however, absent.

Schmitz type.—This variety is similar to *B. dysenteriae* Shiga, but differs in producing indole.

Bacillus dispar.—This type ferments lactose (and sometimes dulcitol) as well as glucose and mannitol. It also forms indole and coagulates milk.

Some of these "atypical dysentery bacilli" correspond to the group designated by Castellani "metadysentery bacilli," which like the classical types are non-gas-producing, but include motile varieties. According to Castellani, these organisms may be associated with a chronic form of dysentery or colitis.

It is difficult to separate the lactose-fermenting types of the "atypical dysentery bacilli" or "metadysentery bacilli" from the organisms classified as *B. coli anaerogenes*, though the latter may occur quite independently of any pathological condition.

Occurrence.—The dysentery bacilli are found in large numbers in the stools at an early stage of the illness, even in practically pure culture, but become progressively less numerous, until, at a later stage, they are apparently absent. They do not invade the blood stream as a rule. The Shiga type is associated mostly with the acute and severe forms of dysentery, the atypical bacilli with the milder cases, and the Flexner-Y type occupies an intermediate position as regards the severity of illness with which it is associated. It may be noted here that the Shiga type differs from the other varieties in its production of a powerful exotoxin.

Concomitants.—When dysentery bacilli tend to disappear from the stool they are found along with, or become replaced by, certain other unusual organisms which may be designated "concomitants."

The commonest are :

B. Morgan No. 1 (<i>Salmonella</i>	} Gram-negative, non-sporing, gelatin-non- liquefying, aerobic bacilli.
<i>morgani</i>) and allied types	
B. "paracolon" types	
B. faecalis alkaligenes (<i>Alcaligenes</i>	
<i>faecalis</i>)	

Their biochemical reactions are shown in the Table on p. 358a.

As *B. faecalis alkaligenes* is a non-lactose-fermenter, its colonies on MacConkey plates may resemble those of *B. typhosus*, the *Salmonella* group, and the dysentery bacilli. They may be relatively large, and around each colony may be noted a broad, light-yellow-coloured zone due to the alteration of the neutral red in the medium by alkali production.

While strains corresponding in general biological characters to this organism have been described with peritrichate flagella, similar organisms are frequently met with which have terminal flagella like a vibrio and show curved vibronic forms. Many strains produce a brown growth on potato like the *B. melitensis-abortus* group, and *B. faecalis alkaligenes* resembles these organisms in its lack of fermentative properties. The biological relationships of this organism are still a matter of doubt.

These organisms are also found in cases of non-dysenteric diarrhoea.

Cases of *B. faecalis alkaligenes* bacteraemia have been recorded.

Morgan's bacillus and allied types are responsible for some cases of infantile diarrhoea in temperate climates.

The *Diagnosis of bacillary dysentery* is dealt with later (p. 352).

THE INTESTINAL PROTOZOA

ENTAMOEBA HISTOLYTICA

The causative organism of one form of tropical dysentery.

Biology.—The vegetative forms are large, rounded, elongated or irregular amoebae, varying in diameter from 10–50 μ , the average size being about 20–30 μ . In the active condition they rapidly protrude and

retract pseudopodia, and appear to move by pseudopod action, but the movements are mainly changes in shape. The cytoplasm consists of a clear hyaline ectoplasm, and a granular, often vacuolated endoplasm. The nucleus is round or oval, and in the unstained condition is not easily distinguished. It is situated in the endoplasm, usually eccentric in position. It is poor in chromatin, and the nuclear membrane is thin. The chromatin granules are small, and are collected in a ring just inside the nuclear membrane. The nucleus shows a small central karyosome. The amoebae ingest red corpuscles, leucocytes and tissue cells, which are observed in the endoplasm, but ingested bacteria are rarely found. The vegetative forms after leaving the body tend to become rounded and immobile, and soon die and disintegrate. Multiplication is by amitotic binary fission.

Under conditions unfavourable to the amoebae—e.g. when the disease is becoming arrested—encystment occurs. Cysts are more or less spherical, with a thin hyaline refractile cyst wall, which gives them a distinct double contour. The contents are finely granular. The average diameter is 9–14 μ . The cysts contain multiple nuclei, *not more than four*, and also thick rod-shaped structures which stain deeply with haematoxylin and are called “chromatoids” or “chromidial bars.” The nuclei contain more chromatin than the nucleus of the vegetative form, and show a distinct karyosome with a central chromatin dot.

The cysts are developed by division of the vegetative form into smaller “precystic” forms. The term “*Entamoeba minuta*” was at one time applied to these precystic forms with nuclei showing a thick ring of chromatin under the nuclear membrane.

Methods of microscopic demonstration and staining are referred to under dysentery diagnosis.

Occurrence.—In the early stage of amoebic dysentery the vegetative forms are present in considerable numbers in the large intestine and in the stools. They penetrate the mucosa of the large bowel and

disintegrate the tissue by their pseudopodia and probably also by means of a liquefying ferment. The sub-mucosa is invaded, and occasionally, small veins from which the amoebae may be carried to the liver. In the bowel, oval or irregular ulcers are developed with undermined edges, which may sometimes lead to perforation of the bowel wall. There is little inflammatory reaction (*cp.* bacillary dysentery) unless a secondary septic infection occurs.

Cysts may be detected in the stools, often in large numbers in chronic cases. After apparent recovery the patient may remain a carrier, and the encysted forms are passed in the faeces. The cyst represents a resting-phase with increased powers of resistance and can survive outside the body for considerable periods. This is the form in which the organism is transmitted from person to person.

The so-called "tropical abscess" results from invasion of the liver probably through the portal circulation. The abscess contains a slimy chocolate-coloured "pus" consisting of necrotic tissue and blood, with only a few leucocytes or pus cells. The amoebae are found mainly in the wall of the abscess, and may not be present in the pus when first evacuated.

Cultivation.—This organism can be cultivated artificially by the method of Boeck and Drbohlav (p. 122).

Experimental Inoculation.—Infection can be produced in kittens by feeding them with material containing cysts; a condition resembling the human disease results. It has been stated that rats can also be infected.

ENTAMOEBA COLI

A non-pathogenic intestinal amoeba which must be carefully differentiated from *E. histolytica* in diagnosis.

The vegetative forms closely resemble those of *E. histolytica*. The cytoplasm is not so clearly differentiated into endo- and ecto-plasm. The pseudopodia are small and delicate and not so refractile as those of *E. histolytica*. The nucleus is usually central in

position, easily distinguishable, rich in chromatin which is often arranged in quadrant form, and has a thick refractile nuclear membrane. The karyosome is well marked. Amoeboid movement is sluggish. Usually no ingested red cells are seen in the cytoplasm (when this organism is noted in a case of dysentery), or, if present, they are not so numerous as in *E. histolytica*. Bacteria are ingested often in large numbers. The cysts are larger ($15-20\mu$) than those of *E. histolytica*, the cyst wall is thick, and there are more than four nuclei—*e.g.* frequently eight—and no chromatoid bodies.

ENDOLIMAX NANA

A frequent non-pathogenic intestinal amoeba. The vegetative form is 8μ in diameter or less. In unstained preparations the nucleus is not distinct, but when stained by haematoxylin it is easily demonstrated, and shows a large irregular eccentric karyosome.

The cysts are oval, and $7-9\mu$ in their longest diameter. They contain one, two, or four small nuclei, but no chromatoid bodies.

IODAMOEBIA BÜTSCHLI AND *DIENTAMOEBIA FRAGILIS* are also included among the intestinal amoebae of man, but need not be described here. Their characters may be ascertained by reference to recent works on protozoology.¹

INTESTINAL FLAGELLATES

These organisms are associated with dysentery and diarrhoea particularly in the tropics, but their pathogenicity is doubtful, and they may occur as commensals.

Trichomonas hominis.—Is pear-shaped, $9-14\mu$ long, and shows a nucleus and cytostome. It possesses three flagella projecting from the broad end, and also another flagellum forming the border of an undulating membrane and with the free part projecting from the pointed posterior end.

Chilomastix mesnili.—Resembles *T. hominis*, but has no undulating membrane and only three flagella. It has an elongated slit-like cytostome. Cysts can easily be recognised; they are oval, about 8μ in their long diameter, and contain one nucleus.

¹ See *Protozoology*, Wenyon, London, 1926.

Giardia or Lamblia intestinalis.—Inhabits the duodenum and jejunum.

Main characters :—somewhat flattened in shape ; flat surface pear-shaped ; bilaterally symmetrical ; $10-18\mu$ in its long diameter ; a large sucking disc on one surface ; two nuclei with karyosomes ; two long median parallel axostyles which represent skeletal structures, with blepharoplasts at each end ; eight flagella in pairs—two arising from the anterior blepharoplasts (the broad end is spoken of as anterior), two arising near the anterior blepharoplasts but following the axostyles to the posterior edge of the sucker before diverging, two arising at the posterior edge of the sucker and rooted in the axostyles, and two arising from the posterior blepharoplasts.

The cysts are characteristic : oval in shape, about $10-15\mu$ long, with two or four nuclei (the cyst containing two organisms formed by subdivision) ; the parallel axostyles are observable.

For further information regarding *other intestinal protozoa*, reference should be made to works on protozoology.

DIAGNOSIS OF DYSENTERY

Collection of specimens of stools.—The stool should be examined as soon as possible after being passed, and should be unmixed with urine. The specimen for laboratory examination is collected in a faeces specimen tube provided with a cork carrying a metal spoon or scoop which fits into the tube, and by means of which faecal matter may conveniently be collected. In the case of a stool containing both faecal matter and mucus, a portion of the latter should be included in the specimen.

Microscopic Examination.—A microscope slide is gently warmed over the Bunsen flame, and on one half of the slide a large drop of normal saline solution is placed and on the other a drop of Lugol's iodine.¹ A loopful of the stool or the mucous discharge is emulsified in the saline drop and another loopful in the iodine solution. The preparations are covered with No. 1 cover-slips, and examined first with the $\frac{1}{6}$ -in. and then with the oil-immersion lens. It is advisable to

¹ This is the iodine solution used in Jensen's modification of Gram's method, *vide* p. 155.

use a "warm-stage" attached to the microscope in examining fresh preparations for amoebae (*vide p. 57*).

Vegetative amoebae can usually be recognised without difficulty. In the saline preparation *Entamoeba histolytica* may often be identified by its active pseudopodial movement and the inclusion in the cytoplasm of numerous red corpuscles (*vide supra*). On the other hand, immobile vegetative amoebae without ingested corpuscles present considerable difficulty in their identification. The presence of cysts facilitates diagnosis owing to the more striking differences between the encysted *E. histolytica* and *E. coli* (*vide supra*). In the iodine preparation the nuclei of the cysts are more distinct than in the saline specimen.

Large phagocytic body cells (macrophages) may be found in dysenteric stools, and are sometimes mistaken for immobile amoebae by inexperienced workers. They often show vacuolation, and may even contain red corpuscles. They are immobile, and the nucleus, unless degenerate, occupying one-fourth or one-fifth of the whole cell, is relatively larger than that of an amoeba, and is not of the ring-like or vesicular type. This distinction is well seen in the iodine preparation. In a heat-fixed film these macrophage cells and their nuclei can be stained with methylene blue, while amoebae cannot thus be demonstrated.

Other intestinal protozoa that may be present can also be detected in unstained preparations (*vide supra*).

Where pathogenic amoebae cannot be detected, the microscopic examination often yields information of diagnostic importance. In a case of bacillary infection there is usually an abundant and characteristic cellular exudate. The cells present are mostly polymorph leucocytes with a varying number of red cells, and in the early stages, numerous epithelial cells. In addition to these, macrophages are frequently a characteristic feature of the exudate. The pus cells as a rule show marked degeneration.

In amoebic dysentery there are few body cells unless

the case is also complicated by a bacterial infection. Leucocytes are scanty, and are not so degenerate as in bacillary dysentery. Charcot-Leyden crystals are frequently seen microscopically in amoebic dysentery and are absent in bacillary dysentery.

The microscopic examination is therefore an important step in diagnosis: the finding of the characteristic *E. histolytica* establishes a diagnosis of amoebic dysentery, while an abundant cell exudate and the absence of amoebae would indicate bacillary dysentery.

This preliminary determination enables a report to be made at once as to the nature of the dysentery, so that the appropriate treatment can be initiated without delay.

Where no amoebae can be found, and if a diagnosis of bacillary dysentery cannot be established later, it is essential that further microscopic examinations be carried out before amoebic infection is excluded.

Stained preparations are of assistance in the identification of intestinal amoebae. Films are made on cover-glasses from the stool and are fixed "wet" by floating the cover-glasses (film downwards) in a fixing solution consisting of 2 parts saturated corrosive sublimate in saline, with 1 part absolute alcohol. They are then stained with iron haematoxylin (as described on p. 175).

Culture.—When the clinical and microscopic data point to a bacillary infection, cultures are made from the stool on MacConkey's medium as in the direct culture of enterica specimens (*vide* p. 332). *In general it may be said that if cases are examined within the first two or three days the infecting organism can be isolated without difficulty.* At a later stage the dysentery bacilli become less numerous and "concomitants" (*vide supra*) are present often in large numbers.

For the complete bacteriological investigation of a case of bacillary dysentery, therefore, it is necessary to consider all the abnormal organisms present in the stool. These are for the most part non-lactose-fermenters, and their colonies are of the "pale" type on MacConkey's medium. Certain varieties of atypical

dysentery bacilli may ferment lactose in fluid medium, but in primary culture from stools present "pale" colonies after twenty-four hours.

Subcultures on agar slopes should be made from each type of pale colony on the MacConkey plate. Since colonies which appear to be similar may represent different organisms, and since there may be, in some instances, even three or four different non-lactose-fermenters present, at least four colonies should be picked off.

Sufficient growth is usually obtained after twelve hours' incubation to proceed with the examination of the agar slope cultures.

Tubes of the following media are inoculated from each culture :—

- | | |
|---------------------------|---|
| (1) peptone water | } with neutral red or
Andrade's indicator and
Durham tube |
| (2) glucose peptone water | |
| (3) lactose | |
| (4) saccharose | |
| (5) mannite | |

(*vide* p. 96)

and these are incubated for twelve to twenty-four hours. The peptone-water culture is examined after six to seven hours for motility of the organisms.

In this way one can ascertain to what group the various cultures belong.

If a culture is obtained with the following characters :—

	<i>Motility</i>	<i>Glucose</i>	<i>Lactose</i>	<i>Saccharose</i>	<i>Mannite</i>
Gram-negative bacillus .	-	±	-	-	- or ±

—*i.e.* corresponding to the classical dysentery bacilli, *B. dysenteriae* Shiga or Flexner-Y (see Table, p. 358a)

—the final identification is made by tests with specific agglutinating sera—*i.e.* an antiserum to the Shiga type, and an antiserum to the classical Y strain or a polyvalent serum prepared by immunising an animal with strains representing the V, W, X and Z antigens.

If the unknown strain corresponds serologically

to one of the classical types, its identification may be regarded as complete. If it fails to react to the appropriate antiserum, its biological characters should be thoroughly studied: dulcitate and maltose media should be inoculated, and the peptone-water tube is tested for the presence of indole (see p. 324) after forty-eight hours, and if negative, also after seven to ten days. The fermentation tubes should be incubated for several days before final readings are made. A gelatin stab, or alternatively a solidified-serum culture, should also be made to test for liquefaction.

If the strain still appears to have the characteristic reactions of a typical dysentery bacillus, the agglutination test should be repeated after the organism has been subcultured several times, as a small proportion of strains are non-agglutinable on first isolation, but rapidly become agglutinable on subculture. If non-agglutinable, it must then be regarded as belonging to the "atypical subgroup" (see p. 346 and Table).

The Sonne type can be identified by its characteristic biochemical reactions and agglutination with a specific antiserum (*vide* p. 347).

Other unusual intestinal organisms (*vide* p. 348) can be identified by completing the cultural tests as in the investigation of the atypical *B. dysenteriae*. Their reactions are shown in the Table on p. 358a.

Agglutination Tests.—In cases where the causative organism cannot be isolated from the stools, agglutination tests have a limited application in diagnosis.

Normal serum may agglutinate the dysentery bacilli in low dilutions (*B. dysenteriae* Shiga in a 1 in 50 dilution, *B. dysenteriae* Flexner-Y in a 1 in 100 dilution), and in cases of bacillary dysentery the specific agglutinating effect of the serum towards the actual causative strain isolated from the case may be relatively weak. Normal serum reactions introduce, therefore, a limitation to the diagnostic test.

As in the case of the Widal reaction, the agglutination reaction is only applicable after five to seven days from the onset of the illness.

In general the test is carried out on the same principle as the Widal reaction (*vide* p. 195); known strains of the different types of *B. dysenteriae* are tested in parallel series with varying dilutions of the patient's serum.

ENTAMOEBA GINGIVALIS

This organism occurs in considerable numbers in pathological conditions of the mouth—*e.g.* pyorrhoea, gingivitis, dental caries—but has no definite aetiological relationship to these conditions. It is about 10–20 μ in diameter and resembles *E. histolytica* in many respects, showing active pseudopodial movement and differentiation of the cytoplasm into ecto- and endo-plasm; the nucleus is indistinct in unstained preparations; the organism apparently possesses the property of ingesting free cells—*e.g.* leucocytes.

THE INTESTINAL ACIDURIC BACTERIA

These organisms constitute a group of acid-resistant (acidophile or aciduric) Gram-positive non-sporing bacilli which occur in the intestine of mammalian animals and are particularly prevalent during the stage of suckling. Thus, in breast-fed infants such organisms may constitute the predominant flora of the intestine, and two main types have been recognised and specially studied.

Organisms of this group are also found in the faeces of various animals, in milk, in the human mouth, stomach and vagina, in soil, and in silage and bran.

BACILLUS ACIDOPHILUS (*Lactobacillus acidophilus*)

So called because it is able to flourish in a highly acid medium. It occurs in faeces, saliva and milk. In morphology it is a relatively large, non-sporing, non-motile, Gram-positive bacillus. The individual bacilli vary in length and may appear even in short coccal forms. Some are about 1 μ broad, but slender forms may be noted, and there is a tendency to chain formation. The organism thus shows considerable pleomorphism. It may be cultivated readily on whey agar at 37° C.

(*vide* p. 122). The colonies are small, and vary in appearance as seen under the low power of the microscope; two main types are described: (1) "feathery," in appearance not unlike a *B. tetani* colony, and (2) rounded with projecting outgrowths ("crab-colony"). A convenient method of obtaining cultures from faeces is to inoculate broth to which is added 0.5 per cent. of glacial acetic acid; after incubation subcultures can be made on agar plates under aerobic conditions.

This organism produces acid fermentation of glucose and lactose without gas formation. It also ferments maltose, whereas *B. bulgaricus*, a related organism originally isolated from Yoghurt (a fermented milk), has no action on maltose. The latter organism cannot grow in the intestine of man. Other allied organisms are *B. acidophilus odontolyticus*, described in association with dental caries, the so-called *Boas-Oppler bacillus* found in the stomach contents in conditions in which hydrochloric acid is absent or deficient, and *Döderlein's bacillus* which is found normally in the vagina.

BACILLUS BIFIDUS (*Bacteroides bifidus*)

Derives its name from the apparently bifid appearance described by the original observers. This organism is found in large numbers in the faeces of breast-fed infants. Its average dimensions are $4-5\mu$ by 0.7μ , but it shows considerable pleomorphism. The ends are often swollen. Three bacilli together may be arranged like a Y. Though usually Gram-positive, there is a certain amount of variation in its reaction to Gram's staining method.

In primary culture it is a strict anaerobe. Cultures have been obtained at 37° C. in tubes of neutral lactose-broth containing a piece of sterile rabbit kidney (*vide* p. 118) with a layer of sterile vaseline superimposed on the medium. After several days' growth, subcultures are made on glucose agar, plates of which are incubated anaerobically. Pure cultures in glucose agar can be obtained from single colonies. The organism may ultimately be grown aerobically.

CHAPTER XV

VIBRIO CHOLERAE AND ALLIED ORGANISMS; BACILLUS PESTIS AND PASTEURELLA GROUP

VIBRIO CHOLERAE (*Vibrio comma*)

THE causative organism of Asiatic Cholera.

Morphology.—Curved or “comma-shaped” rods (vibrios) with rounded or slightly pointed ends, about $1.5\text{--}3\mu$ by 0.5μ . They are actively motile, and the movement is of a “darting” or “scintillating” type. Motility is due to a single long terminal flagellum. The vibrios occur singly, in pairs, or in chains end to end with the curves alternating—*i.e.* presenting a somewhat spiral arrangement. “S” forms and spirals representing elongated undivided single cells may be noted. Involution occurs readily, especially in culture, and globular, club-shaped or irregular forms may be observed. No spores are produced.

When the organism has been growing in artificial culture for a time, the morphology becomes less typical and the curvature of the vibrios is less pronounced.

Staining.—Gram-negative.

Culture.—Aerobe; slight growth also occurs under anaerobic conditions. Temperature range— $16^{\circ}\text{--}40^{\circ}\text{C.}$, optimum— 37°C. Grows on ordinary media. A slight trace of acid is inhibitory, and abundant growth occurs on even highly alkaline media—*e.g.* Dieudonné’s medium (*q.v.*). The optimum reaction is pH 8.0–9.0.

Colonies on agar—white circular discs about the size of *B. coli* colonies, semi-transparent, with well-

defined circular margins; older growths develop a brownish-yellow colour.

Other types of colony may be noted as variants from the standard form—*e.g.* an opaque yellowish-white colony, and a “ring” colony with an opaque centre and transparent border.

Gelatin stab—at first there is a white line of growth along the needle track; then liquefaction occurs at the top and spreads downwards in funnel-shaped form.

Coagulated serum is liquefied.

Potato slope—at first, white layer of growth forms, and later a brownish-yellow or pinkish colour is developed; to elicit this growth the reaction of the potato medium must be alkaline.

In broth—a uniform turbidity results, and a characteristic pellicle forms on the surface.

Biochemical Reactions.—The fermentative reactions are as follows:—

Glucose	Lactose	Dulcitate	Saccharose	Mannite	Maltose
+	-	-	+	+	+
	(some strains l. after several days' growth)				

(+ = acid; no gas)

They can be tested for as in the case of the colityphoid group.

Cholera-red reaction—this depends on the production of indole *and nitrites* in peptone water. It can be elicited by adding a few drops of sulphuric acid to a four-days' peptone-water culture. A reddish-pink colour develops, due to the formation of nitroso-indole.

Haemolysis—the classical type of *V. cholerae* is *non-haemolytic*, but haemolytic vibrios showing a serological relationship to *V. cholerae* may be met with in cholera-like cases (*e.g.* the “El Tor vibrio”).

It is advisable to test for haemolysis by adding varying amounts (0.1–1.0 c.c.) of a three-days' broth culture to 1 c.c. of a 5 per cent. saline suspension of sheep's red blood corpuscles in tubes which are incubated for two hours and then allowed to stand overnight in the ice-chest; observations are then made (Greig).

Haemolytic vibrios, such as the El Tor variety, manifest their haemolytic action when growing on blood-agar, but certain vibrios, including strains of *V. cholerae* which do not form haemolysin, also produce clearing or apparent laking when growing on this medium, due probably to a chemical alteration of the haemoglobin. Such vibrios clear coagulated blood media—e.g. “chocolate agar”—in the same way as ordinary blood-agar.

Serological Reactions.—From the serological standpoint the cholera vibrio is generally regarded as a homogeneous species, and unknown strains can be identified by testing their agglutination reaction with an antiscrum to a known *V. cholerae*.

The organism possesses both H and O antigens, and certain antigenic constituents of either the H or O type may be shared with other vibrios—e.g. the “El Tor vibrio” (*vide supra*), which differs from the classical cholera organism in its haemolytic property. Agglutinin-absorption tests may be required to differentiate these (Gohar).

Viability.—*V. cholerae* is killed at 56° C. in thirty minutes. It dies within two or three hours when subjected to drying. In stagnant water the organism may survive for a considerable period—e.g. eighteen days or longer. While the cholera vibrio may be distributed by water supplies, it is doubtful whether it can exist outside the body as a saprophyte.

Occurrence.—Typical cholera is an acute disease of sudden onset, characterised by intense diarrhoea and tenesmus, vomiting, “rice-water” stools, muscular cramps, and extreme collapse.

The vibrios multiply in large numbers in the lumen of the small intestine and are present in large numbers in the intestinal contents and dejecta. There is great epithelial desquamation, and the stools contain white flakes consisting of epithelial cells and mucus. Numbers of vibrios are demonstrable in these flakes. The organism does not penetrate deeply in the bowel wall and practically never invades the blood stream. The gall bladder is frequently infected.

Convalescents may remain carriers, and the organism is apparently retained in the gall bladder.

DIAGNOSIS

The bacteriological diagnosis depends on the isolation and identification of the vibrio.

The organism may be detected microscopically in the intestinal dejecta, but this is not sufficient for accurate laboratory diagnosis, and inexperienced workers may easily be misled by slightly curved bacilli simulating vibrios.

PROCEDURE FOR CULTURE AND ISOLATION

I.—(a) A tube of 10 c.c. peptone water is inoculated with a flake of mucus from the stool, or, in the case of a fluid faecal stool, with a large loopful of the specimen. In examining possible carriers, the stool, if solid or semi-solid, is thoroughly emulsified in sterile salt solution and several loopfuls are added to the medium. The peptone water used is a 1 per cent. peptone with 0.5 per cent. sodium chloride, standardised to pH 8.4—*i.e.* distinctly alkaline. The tube is incubated for six to eight hours. Within this time vibrios, if present, grow freely and at the surface of the medium, and even outgrow other intestinal bacteria.

(b) A plate of Dieudonné's medium (*vide* p. 118) is also inoculated directly from the stool, and incubated for twelve to eighteen hours. This medium is highly alkaline and is selective for vibrios, inhibiting the growth of most other intestinal bacteria. A practically pure culture of an intestinal vibrio can be obtained in this way from a stool containing large numbers of *B. coli*.

II. The peptone-water culture is examined after six to eight hours by means of stained films made from a drop of the surface layer of the culture : a large loopful is placed on a slide and, without spreading it, slowly dried at room temperature ; the film is then fixed by heat, and washed in a stream of water to remove the dried peptone particles which stain deeply and obscure the organisms ; the preparation is stained with dilute carbol fuchsin for one minute and examined microscopically. At the same time a

hanging-drop preparation is examined; at the edge of the drop, vibrios are easily detected by their characteristic morphology and "scintillating" or darting motility. In general, however, the fuchsin-stained film can be relied on alone for the detection of vibrios. If vibrios are present, a sub-inoculation is made on a Dieudonné plate. If no vibrios are detected, a sub-inoculation is made into a second peptone-water tube; this tube is incubated for six to eight hours, and a film from it is then examined as in the case of the primary culture: if vibrios are present, a Dieudonné plate is inoculated from the peptone culture. If no vibrios are detectable in the second peptone-water culture the result may be regarded as negative.

Where vibrios are relatively less numerous, and are not cultured directly on a Dieudonné plate, the organism can be isolated after enrichment in either one or two peptone-water cultures.

Pure cultures on agar slopes are obtained from isolated colonies on the Dieudonné plates; the morphological, cultural and biochemical characters of the strains are then determined; the final identification of the organism depends on its agglutination by a specific anti-cholera serum obtained by immunising an animal with a known *V. cholerae* (*vide*. 200).

If a direct growth from the stool is obtained on Dieudonné's medium it is, as a rule, practically pure, and the bacteriological diagnosis can be expedited by carrying out the agglutination reaction at once without waiting for the results of cultural tests. Thus within eighteen to twenty-four hours it is possible to determine whether the condition is due to *V. cholerae*. At the same time also it is advisable to obtain cultures from single colonies and confirm the identity of the organism by detailed tests.

ISOLATION OF VIBRIO CHOLERAE FROM WATER

100 c.c. of a sterile alkaline 10 per cent. solution of peptone containing 5 per cent. sodium chloride are added to 900 c.c. of the water specimen, which is then distributed in sterile stoppered

flasks. These are incubated, and sub-inoculations are made (from the surface growths) on Dieudonné's medium after twenty-four and forty-eight hours, as in the method described above for the isolation of *V. cholerae*.

THE PARACHOLERA VIBRIOS

These are associated with choleraic conditions, usually of lesser severity than true cholera, and occurring as sporadic cases or in limited outbreaks.

Their morphological, cultural and biochemical characters generally correspond to those of *V. cholerae*, but most of the types described are haemolytic, like the El Tor vibrio (*vide supra*).

They do not react to an agglutinating anti-cholera serum, and represent a number of serological types, differing in their agglutination reactions with antisera.

The bacteriological diagnosis in paracholera is carried out in the same way as in true cholera.

OTHER VIBRIOS

Two species of vibrios have been described in diseases of animals—*V. metchnikovi* and *V. foetus*.

V. metchnikovi was first isolated from a septicæmic disease of fowls. It resembles closely *V. cholerae* in general biological characters, but differs serologically, and in its high virulence for guinea-pigs, pigeons and fowls: a minute amount of culture introduced intramuscularly or into a cutaneous wound produces in these animals a rapidly fatal septicæmia. *V. cholerae* does not show such degree of pathogenicity. Similar organisms have been isolated from choleraic cases and from water.

V. foetus has been reported in abortion of sheep and cattle, and isolated from the placenta and also from the foetus. The organism varies in length, the short forms being comma-shaped, the longer individuals exhibiting two to four coils. It is relatively slender and shows a flagellum at one or both ends. It stains Gram-negatively. This organism is micro-aerophilic and has been cultivated on agar slopes in sealed tubes containing in the condensation water a few drops of sterile defibrinated horse blood. Growth is not abundant, and is most marked in the condensation water. Laboratory animals are not susceptible to experimental inoculation.

Various other vibrios and spirilla have also been described. These are mostly water forms, but some have been isolated from other sources—e.g. from sputum (*V. sputigenus*), from

cheese (*V. tyroginus*), from intestinal contents in "Cholera nostras" (*V. proteus*—Finkler and Prior's spirillum). Certain water vibrios exhibit in culture marked phosphorescence—e.g. *V. phosphorescens*. This type of organism has also been found in choleraic cases.

BACILLUS PESTIS (*Pasteurella pestis*)

The organism of Oriental Plague.

Morphology and Staining.—In its most characteristic form this organism is a short, oval bacillus with rounded ends—i.e. cocco-bacillary—about 1.5μ by 0.7μ , occurring singly and in pairs. In the tissues a capsule may be observed, and in cultures grown at 37°C . a gelatinous envelope has been demonstrated by means of Indian-ink preparations.

It is Gram-negative, and when stained with a weak stain (e.g. methylene blue) shows characteristic bipolar staining, which is an important feature in identification.

In culture the plague bacillus is less typical. Longer forms are frequent, and polar staining is less obvious. Pleomorphism is marked especially in old cultures, and involution or degeneration forms are particularly noticeable. These are markedly swollen, stain faintly, and include globular, pear-shaped, elongated or irregular forms. In fact the microscopic picture of an old culture often suggests that of a yeast or mould. Involution in culture can be hastened by the presence of 3 per cent. sodium chloride, and this has been utilised in identifying the organism.

In fluid culture the bacilli tend to be arranged in chains.

The organism is non-motile and non-sporing.

Culture.—Grows aerobically on ordinary culture medium. Unlike other pathogens, the optimum temperature is 30° – 35°C ., and primary cultures grow best at even lower temperatures—e.g. 27°C . The minimum temperature is about 14°C .

Colonies on agar—at first very small transparent white circular discs (1 mm. or less), later becoming

larger (3–4 mm.) and opaque; they are not specially characteristic.

In older cultures some of the colonies may have outgrown the others and become more opaque. This appearance is not unlike that of a mixed growth.

Gelatin stab—no liquefaction occurs.

Broth—growth consists of a granular deposit at the foot and on the sides of the tube, not unlike that of a streptococcus. If cultured in a flask of bouillon with drops of sterile oil on the surface, and provided the flask is not subjected to shaking or movement, a characteristic growth develops, consisting of “stalactites” hanging down into the fluid from the oil drops.

Biochemical Reactions.—

Glucose	Lactose	Dulcitol	Saccharose	Mannitol	Milk	Indole
+	—	—	—	+	—	—

(+ acid; no gas)

Growth occurs on a bile-salt medium—*e.g.* MacConkey's (*cp.* other members of the *Pasteurella* group).

It should be noted that the risk of laboratory infection from handling pathological material and cultures is considerable, and all manipulations should be carried out with the utmost care.

Antigens.—It has been shown that *B. pestis* contains two types of antigen, one somatic and heat-stable, the other heat-labile at 100° C. and associated with the gelatinous envelope which is formed in cultures growing at 37° C. (*vide supra*). The envelope antigen is of importance in relation to the immunising properties of *B. pestis* vaccines: thus a vaccine prepared from cultures grown at 37° C. in which the envelope is well developed possesses greater immunising properties than from cultures grown at lower temperatures—*e.g.* 25°–30° C. as in the preparation of the Haffkine plague vaccine generally used in India (Schütze). Heating the culture for half-an-hour at 56° C. before addition of phenol does not affect the antigenic value of the resulting vaccine.

Viability.—The thermal death-point is about 55° C. Dies when desiccated within one to two days. Laboratory cultures remain viable for long periods (*e.g.* months) if kept moist and at low temperatures.

Animal Inoculation.—The bacillus is pathogenic to monkeys, rats, guinea-pigs and other rodents, and plague is essentially an epizootic disease among wild rats and certain other rodent animals. A guinea-pig or white rat injected subcutaneously with a recently isolated culture dies in a few days, and at autopsy a marked local inflammatory condition is noted, with necrosis and oedema; the related lymph glands are also involved; the spleen is enlarged and congested and often shows small greyish-white areas in its substance; there is also septicaemia. The characteristic bacilli can be seen in large numbers in films from the local lesion, lymph glands, spleen pulp and heart blood. A similar condition is found in rats dying of epizootic plague (*vide infra*).

Rats and guinea-pigs can be successfully inoculated by applying infected material to a shaved area of skin or to a mucous membrane—e.g. of the nose.

Occurrence in Human Lesions.—In *Bubonic Plague* the bacilli are present in large numbers in the affected lymph glands. When the bubo undergoes necrosis as the condition advances, they become less numerous, and may even disappear. Septicaemia may result, and then the bacilli can be detected in the blood during life by blood culture. Post-mortem they are found in the spleen.

In *Pneumonic Plague* the bacilli are present in large numbers in the sputum and in the broncho-pneumonic areas in the lung.

In *Septicaemic Plague* the condition is a general infection without definitely localised lesions.

Plague is epizootic in rats and certain other rodents. The infection is spread by rat fleas (e.g. *Xenopsylla cheopis*). The occurrence of bubonic plague in man is due to transmission of the infection from rats by the same agency. The mechanism of transmission is as follows:—The flea sucks blood (containing plague bacilli) from an infected animal; the bacilli multiply in the stomach and proventriculus, which may become blocked with bacillary masses; when the insect again bites and sucks blood,

regurgitation takes place from the blocked proventriculus into the bite wound, and so inoculation results.

The time during which a flea remains infective—*i.e.* the period of survival of the bacilli—depends on temperature and humidity. A temperature of about 10° C. and a high degree of humidity have been found by experiment to be the most suitable conditions.

Pneumonic plague is communicated from person to person by infected secretion droplets from the respiratory passages.

DIAGNOSIS

Bubonic Plague.—The bubo is punctured with a hypodermic syringe and exudate withdrawn. From this material, films are made and stained with methylene blue and by Gram's method. The appearance of the characteristic bacilli showing bipolar staining is highly suggestive.

Cultures are also made on nutrient agar, and single colonies are picked off on to agar slopes. The resulting cultures are then available for further investigation.

Some of the exudate should also, if possible, be injected subcutaneously into a guinea-pig or white rat. If plague bacilli are present, the inoculated animal will die, showing at autopsy the appearances, etc., described above.

The cultures obtained may be tested as regards: biochemical reactions, involution on 3 per cent. salt-agar, chain formation in bouillon, and stalactite growth. The cultures can also be used for further animal-inoculation experiments.

Pneumonic Plague.—The bacilli can be detected microscopically in the sputum, and for identification should be isolated in pure culture as in dealing with material from bubonic plague.

In carrying out animal inoculation with sputum, other virulent organisms may be present (*e.g.* pneumococci); instead of injecting subcutaneously, successful inoculation with *B. pestis* can be effected by applying the material to the nasal mucosa, or to a shaved area of skin.

In septicaemic plague, the bacillus can be demonstrated and isolated by blood culture (*vide* p. 141).

DIAGNOSIS OF PLAGUE INFECTION IN WILD RATS

At autopsy the following appearances are noted :—enlargement of lymphatic glands, with periglandular inflammation and oedema, most frequently in the cervical glands owing to the fact that the neck is the common harbourage of fleas ; serous effusion in the pleural cavity ; enlargement of the spleen, which may show small white nodules in the pulp ; congestion and a mottled appearance of the liver ; congestion and haemorrhage under the skin and in the internal organs.

Films are prepared from the heart blood, the glands and spleen, and stained by Gram's method and with methylene blue. Cultures should also be made, and the isolation of the organism attempted by the usual methods. Guinea-pigs should be inoculated subcutaneously with an emulsion of the splenic tissue. In rats found dead of plague it may be difficult to demonstrate the bacilli microscopically or to isolate them in culture. Carcasses in a state of decomposition may be heavily contaminated with other organisms which render the microscopic examination confusing and isolation difficult. Infection of a white rat or guinea-pig, by smearing the nasal mucous membrane or a shaved area of skin with material from the lesions, should be carried out.

PASTEURELLA GROUP

The plague bacillus is only one species in a large biological group (the *Pasteurella* group) which includes the organisms of "haemorrhagic septicaemia" in various animals and *B. pseudo-tuberculosis rodentium*. These organisms and *B. pestis* all show a similarity in morphology, staining reactions, cultural and biochemical characters, but differ in certain characters and in their parasitism and virulence to different animal species.

Bacillus avisepticus (*P. aviseptica*) is the causative organism of "fowl cholera" and septicaemia in certain birds. In the blood the bacilli are present usually in considerable numbers and show characteristic bipolar staining. Under experimental conditions this organism is virulent to fowls, pigeons and rabbits, but in guinea-pigs the infection may remain localised to the site of inoculation. (It may be noted here that Pasteur's classical work on attenuation of virulence by continued cultivation for purposes of artificial immunisation was carried out with this organism.)

Bacillus suisepiticus (*P. suisepitica*). The organism of swine plague which takes the form of a rapidly fatal septicaemia

or a pneumonia with enteritis. Various animals are susceptible to experimental inoculation—*e.g.* swine, rabbits, cats, cattle, sheep; guinea-pigs and pigeons are stated to be less susceptible. This type of organism has been noted by various observers as a commensal in the respiratory passages of swine, and the question whether it is the primary causal agent of swine plague is not entirely settled.

Bacillus boviseppticus (*P. bovisepptica*). The organism of haemorrhagic septicaemia of cattle and of septic pleuropneumonia of calves. On experimental inoculation this organism is highly pathogenic to cattle, rabbits and mice.

Other recognised organisms of the group are:—

P. cuniculicida (or *B. lepiseppticus*) of rabbit septicaemia and snuffles (*vide* p. 193), *P. equiseptica* (of haemorrhagic septicaemia in horses), *P. pseudo-tuberculosis rodentium* (occurring in pseudo-tuberculosis and septicaemia of rats and certain other rodents). Similar infections also occur in sheep, goats, dogs etc.

These organisms of haemorrhagic septicaemia can be differentiated from *B. pestis* by the inhibition or absence of growth on a taurocholate medium (*e.g.* MacConkey's) on which the plague bacillus is able to grow. *P. pseudo-tuberculosis rodentium*, which might be confused with *B. pestis*, if isolated from wild rats can be distinguished by its motility when growing at 22° C. and its feeble pathogenicity to white rats.

Three serological types of *B. pseudo-tuberculosis rodentium* have been recognised. The organism possesses three antigenic constituents: (1) flagellar, (2) somatic and type-specific, and (3) somatic and common to the different types. This last is the same as the somatic antigen of *B. pestis*.

The following Table shows how *B. pestis*, *B. pseudo-tuberculosis rodentium* and *B. avisepticus* (representing the haemorrhagic septicaemia subgroup) may be differentiated:

	Motility at 22° C.	Saccharose	Milk	Indole	Growth on Bile-salt Media	Pathogenicity to White Rat
<i>B. pestis</i> . . .	—	—	No change	—	+	+
<i>B. pseudo-tub. rodentium</i> .	+	$\perp$	Alk.	—	+	—
<i>B. avisepticus</i> .	—	$\perp$	No change	+	—	+

B. avisepticus is taken as representative of the various organisms which have been described in haemorrhagic septicaemia of animals.

BACTERIUM TULARENSE

In the Western States of America a plague-like disease occurs in wild rodents (ground-squirrels and jack-rabbits) due to an organism designated *Bacterium tularense*. The pathological lesions are not unlike those found in plague-infected animals, and this infection has to be considered, therefore, in the diagnosis of plague in animals. The disease has also been observed in Japan, Russia and Norway. Various rodent animals may be infected. The organism is a small rod-shaped structure not usually exceeding 0.7μ in length, and sometimes capsulated. It is present in large numbers in the spleens of infected animals, but cannot be cultivated like the plague bacillus on ordinary media. Cultures have been obtained on a medium consisting of pure egg yolk, on blood-agar and serum-agar containing a piece of sterile rabbit spleen, and on agar containing 0.02 per cent. cystine.

This organism shows serological relationships to the *B. melitensis-abortus* group.

This infection is also transmissible to man—*e.g.* from handling infected animals, and from laboratory cultures (which retain an *extremely high degree* of infectivity). A prolonged febrile illness results, sometimes with glandular lesions and ulcers of the skin. The serum of infected persons agglutinates the organism. For diagnostic purposes guinea-pigs or mice may be inoculated with exudate from the glands or ulcers.

CHAPTER XVI

BACILLUS MELITENSIS AND BACILLUS ABORTUS; HAEMO- PHILIC BACTERIA (BACILLUS INFLUENZAE, ETC.); BACTERIUM PNEUMOSINTES

BACILLUS MELITENSIS (*MICROCOCCUS MELITENSIS*, *Brucella melitensis*, *Alcaligenes melitensis*)

THE causative organism of Malta or Undulant Fever.

Morphology.—A cocco-bacillus, usually appearing as round or oval forms about 0.4μ in diameter. Definite bacillary forms ($1-2\mu$ in length), however, may be observed. The organisms occur singly, in pairs, or even short chains. It is non-motile and non-sporing.

Staining.—Gram-negative.

Culture.—Grows under ordinary aerobic conditions. Optimum temperature— 37° C. Grows even at 20° C. It can be cultivated on ordinary nutrient media, but a better growth is obtained on serum media.

Colonies on agar—in primary growth may not appear for three or four days; they are small transparent discs about 1 mm. in diameter, but increase in size to about 3 mm.

Gelatin stab—a delicate line of growth along the needle track with little or no surface growth. No liquefaction occurs.

On potato medium a greyish or brownish growth is noted.

B. melitensis exhibits no fermentative properties.

Viability.—The thermal death-point is about 60° C. Resists drying for two to three months. Laboratory cultures remain viable for several months.

Occurrence.—The organisms are present in the blood, especially at an early stage. In some cases (about 10 per cent.) they may be demonstrated in the urine. Post-mortem they are found in considerable numbers in the spleen and also in various organs. Infection results usually from the ingestion of the milk of infected goats. Where the disease is endemic (*e.g.* in Malta and the Mediterranean littoral) a considerable percentage of goats are infected, and the organisms can be demonstrated in the milk.

A "*B. paramelitensis*" has been recognised differing from *B. melitensis* in agglutination reactions with antisera, though otherwise similar. It is possible that this type is a "rough" variant of *B. melitensis*.

DIAGNOSIS

Blood Culture should be carried out in all cases, and it is essential that at least 10 c.c. of blood should be withdrawn for this purpose, as the organisms may be relatively scanty. Cultures may also be obtained by spleen puncture.

In some cases the organism may be isolated from the urine.

The agglutination test with patient's serum and a known strain of *B. melitensis* is carried out as a routine procedure. The reaction may be elicited after five days from the onset of the illness. It has to be noted that normal serum may agglutinate *B. melitensis* in dilutions even up to 1 in 100, and this introduces a possible fallacy in the application of this diagnostic test. In cases of undulant fever, however, the serum often agglutinates *B. melitensis* in high dilutions—*e.g.* 1 in 1000. If, in carrying out the agglutination test in a suspected case, the reaction occurs only with low dilutions (*e.g.* 1 in 100), the result cannot be regarded as conclusive. If the test is repeated, a "rising titre" may be observed and a more conclusive result obtained.

Post-mortem, it can be cultured from the spleen.

In goats, the infection can be recognised by using

the agglutination test with the animal's serum and by cultivating the organism from the milk.

BACILLUS ABORTUS (*Brucella abortus*, *Alcaligenes abortus*)

The organism of bovine contagious abortion; occurs also in pigs and sheep. In morphology and general biological characters this organism closely resembles *B. melitensis*. Two types have been recognised, *bovine* and *porcine*, these being the varieties commonly found in cattle and pigs respectively. The *porcine* type resembles *B. melitensis* even more closely than the *bovine* type.

When cultivation is attempted directly from the animal body, the *bovine* type does not grow under ordinary aerobic conditions and requires an atmosphere containing 5–10 per cent. of carbon dioxide. This can be obtained by placing the inoculated tubes or plates in an air-tight jar, exhausting it to the required degree and replacing 5–10 per cent. of the air with carbon dioxide. After continued cultivation, however, the organism may be grown in the ordinary atmosphere. The *porcine* type, like *B. melitensis*, can be grown under the usual aerobic conditions and is not dependent on such a high carbon dioxide content in the atmosphere as the *bovine* form. This is an important distinguishing feature. The most suitable medium is a liver-infusion or liver-digest "hormone" medium (*vide* p. 88); the addition of gentian violet in a concentration of 1 : 250,000 facilitates isolation of the organism from material likely to contain other bacteria. Bang originally cultivated the *bovine* type by preparing shake cultures in tubes of serum-agar, the colonies developing best in a zone just below the surface of the medium. A convenient method of producing a suitable atmosphere for the growth of this organism in a tube is simply to seal the mouth of the tube with paraffin after flaming the stopper. This procedure usually results in a sufficiency of carbon dioxide in the contained air.

On potato medium *B. abortus* produces a brown

growth like that of *B. melitensis*; it exhibits no obvious fermentative reactions. Both the *bovine* and *porcine* types form sulphuretted hydrogen (the latter more markedly) which is not a property of *B. melitensis*. This can be tested for by placing a piece of moistened lead acetate paper at the mouth of the culture tube and replacing it, if necessary, daily for five days. The *porcine* type has been differentiated from the *bovine* by means of media containing 1 : 30,000 thionin and 1 : 25,000 basic fuchsin respectively. *B. melitensis* is not inhibited to any extent by these dyes, the *porcine* type is inhibited by fuchsin but not by thionin, whereas the *bovine* type is inhibited by thionin not by fuchsin.

The differences between the three types may be summarised as follows :

	CO_2 requirement	H_2S production	Growth in presence of	
			Basic fuchsin 1:25,000	Thionin 1:30,000
<i>B. melitensis</i> . . .	—	—	+	+
<i>B. abortus</i> bovine type. . .	+	+	+	—
porcine type . . .	—	++	—	+

B. abortus has been isolated from the stomach contents, heart blood and tissues of the aborted foetus and from the uterine exudate, and in such materials it may also be demonstrated microscopically by appropriate methods. It may be present in the udder and excreted in the milk. Its specific relationship to the disease has been established by the experimental production of abortion in pregnant animals following intravenous injection of cultures or inoculation into

the vagina. Similar experimental effects have also resulted from administration of cultures by the mouth.

Experimental inoculation of cultures into guinea-pigs may produce tubercle-like lesions (*e.g.* in lymph glands, spleen, liver, etc.), and as *B. abortus* may occur in cow's milk, this has to be remembered in relation to the animal inoculation test for tubercle bacilli (*vide p.* 240).

Strains of *porcine* type are more virulent for guinea-pigs than those of *bovine* origin.

The *bovine* type of *B. abortus* is less virulent to monkeys than *B. melitensis*, which produces under experimental conditions in these animals a condition analogous to undulant fever. The *porcine* type resembles *B. melitensis* in its virulence for monkeys.

A "*B. para-abortus*" has been described analogous to *B. paramelitensis* (*vide supra*). It is probably an R variant of *B. abortus*.

Diagnosis.—The agglutination test with the serum of supposed infected animals and known cultures of *B. abortus* has been used in diagnosis. The serum of normal animals may, however, exert some degree of agglutinative effect, and only results in which agglutination occurs in dilutions of 1 in 40 or over can be accepted as definitely positive.

In animals that have aborted the organism can be demonstrated microscopically in the uterine discharge and also in the stomach contents of the foetus and can be cultivated by the methods referred to above. Inoculation of a guinea-pig may be resorted to for demonstrating and isolating the organism; the inoculated animal is killed after four weeks and cultures are made from the spleen. The inoculation test is also utilised for demonstrating the organism in milk.

RELATIONSHIP OF *B. ABORTUS* TO *B. MELITENSIS* AND ITS OCCURRENCE IN UNDULANT FEVER

B. abortus and *B. melitensis* show a very close biological relationship, and there has been uncertainty whether they can be clearly differentiated by serological reactions. Direct

agglutination tests with antisera differentiate *B. melitensis* from *B. paramelitensis* and likewise *B. abortus* from *B. paraabortus*, but such tests fail to distinguish between *B. melitensis* and *B. abortus* and between *B. paramelitensis* and *B. paraabortus*. Agglutinin-absorption tests however elicit a difference between *B. melitensis* and *B. abortus*. The difference in the antigenic constitution of the two organisms is probably quantitative rather than qualitative.

It is now well established that *B. abortus* may, under certain conditions, produce undulant fever ("abortus fever") in man, infection being derived from cow's milk, or through contact with cattle or pigs; thus strains of *porcine* origin as well as *bovine* strains may be responsible for human infections. *B. abortus* may occur in a considerable percentage of samples of unsterilised market milk, but the incidence of "abortus fever" is exceedingly low. Latent infections, however, are not uncommon. It would appear, therefore, that the pathogenicity of the *bovine* strains of *B. abortus* must be comparatively slight for the human subject. The *porcine* strains on the other hand possess a higher virulence. The infection in man may be either a typical "Malta fever" or an irregular febrile illness which is mild in type. The serum of these cases agglutinates *B. abortus*, often in high dilutions, and the organism may be cultivated from the blood.

B. bronchisepticus, an organism described in canine distemper, but only a secondary invader, is biologically related to *B. melitensis* and *B. abortus*. It differs from these organisms in being motile and possessing peritrichate flagella.

BACILLUS INFLUENZAE AND THE HAEMOPHILIC BACTERIA

Bacillus influenzae, originally described as the causal organism of epidemic influenza, has been designated "haemophilic" in virtue of its inability to grow on culture medium without the addition of whole blood or certain growth-promoting substances present in blood: (1) haematin, which acts like a peroxidase, and (2) a vitamin. These growth factors, however, are not restricted to blood, but are present also in certain vegetable tissues. The Koch-Weeks bacillus (*vide* p. 264) shows the same growth requirements as *B. influenzae*, and may appropriately be

grouped with it. *B. pertussis* (of whooping cough) has also been placed in the "haemophilic" group, but its growth requirements are entirely different from those of the influenza bacillus. Similarly the bacillus of Morax (*vide* p. 264) and Ducrey's bacillus (*vide infra*) have been included by some systematists in the "haemophilic group" along with *B. influenzae*, but such grouping is not strictly justifiable. If the term "haemophilic" is used in a broad sense to designate organisms which require blood for their growth it would embrace a number of heterogeneous species, and it is preferable to restrict the term to those organisms which are dependent on the growth factors required by *B. influenzae*.

BACILLUS INFLUENZAE (PFEIFFER)

(*Haemophilus influenzae*)

Morphology.—A very small slender bacillus, usually about 1.5μ by 0.3μ , with rounded ends, occurring singly or in pairs; non-motile; non-sporing. Shorter oval cocco-bacillary forms are also noted, and in culture there is marked pleomorphism. Some strains, particularly those present in meningitis, show elongated curved thread-like forms.

Staining.—Gram-negative; carbol fuchsin in a 1 in 20 dilution should be used as the counter-stain and applied for five minutes.

Culture.—Aerobe. Optimum temperature—about 37°C . Does not grow on ordinary media but can be cultivated in the presence of blood—therefore classified as "haemophilic"—*e.g.* on blood-agar or preferably heated-blood-agar (*vide* p. 108).

It has been shown that two growth-promoting constituents are necessary for the cultivation of *B. influenzae*. One of these, termed the X factor, is thermostable and resists autoclaving at 120°C . It is associated with haemoglobin, and reacts like a peroxidase, accelerating the transfer of oxygen from peroxides. This factor is essential for the oxidative

processes of the organism. The other factor, designated V, is more easily destroyed by heat, and is probably of the nature of a vitamin. Both factors are present in blood. Certain plant tissues—*e.g.* potato—contain both the V factor and a substance corresponding to the X factor which acts as a peroxidase.

On blood-agar—very small transparent droplet-like colonies which tend to remain discrete.

It is noteworthy that *B. influenzae* grows better in symbiosis with staphylococci, etc. This is due to the fact that these organisms synthesise the V factor.

Glucose and various other carbohydrates are fermented. Indole is produced by some strains.

Haemolytic types.—Certain strains classified as *B. influenzae* have been found to differ from the typical form in their haemolytic properties and these are sometimes coarser in microscopic structure. They also tend to develop elongated threads. While the typical influenza bacillus requires for its growth both the X and V factors, most of these haemolytic strains require only the V factor.

B. haemoglobinophilus canis, an organism originally isolated from a purulent condition of the preputial sac in a dog, resembles *B. influenzae* in general characters, but requires only the X factor for its growth.

Experimental Inoculation.—It has not been found possible to produce in laboratory animals a condition corresponding to influenza by experimental inoculation with this organism. According to certain observers a soluble toxin is formed in culture which on inoculation in laboratory animals produces pulmonary lesions like those in epidemic influenza.

Occurrence.—Found in the sputum, nasal and throat secretions in a considerable proportion of cases of epidemic influenza with inflammatory conditions of the respiratory system. In the inflammatory exudate the bacilli are often present inside leucocytes—*i.e.* intracellular. In the sputum they may occur in exceedingly large numbers, but usually along with the pneumococcus, streptococci and other organisms associated

with inflammation of the respiratory passages. They may be found in the pulmonary lesions in influenzal pneumonia, in empyema and other complications, and have also been noted in the blood in some cases.

It must be remembered that organisms of the type of *B. influenzae* may occur in catarrhal conditions of the respiratory system *apart altogether from epidemic influenza*, and similar organisms may occur in the mouth and throat in healthy persons.

This organism cannot be regarded as the specific primary cause of epidemic influenza, though it may play an important part in the pathogenesis of the pulmonary complications. Its rôle is probably a secondary one.

Haemophilic strains (*vide supra*) have also been found in cases of meningitis occurring in young children.

B. PERTUSSIS (*Haemophilus pertussis*)

Originally described as the causative organism of whooping-cough.

Morphology and Staining.—A very small oval coccobacillus, slightly larger than *B. influenzae*, often showing polar staining; definite bacillary forms are noted, but it is generally more oval in form than *B. influenzae*; non-motile; non-sporing; Gram-negative.

Culture.—Strict acrobe. Compared with *B. influenzae*, blood is not so necessary for growth. It is usually cultivated on blood media; in subculture, however, it may grow on media containing serum but without blood pigment, and is independent of the X and V factors required by the influenza bacillus. For primary culture the special medium of Bordet and Gengou (p. 117) should be employed. The colonies develop slowly, and are larger, thicker and more opaque than those of *B. influenzae*, and have also a "pearly" appearance; growths may be sticky or slimy.

The organism has no fermentative properties. The species is serologically homogeneous.

B. pertussis differs also from the influenza bacillus in its continued viability at low temperatures (0° – 10° C.) and its inability to grow, when first isolated, on agar containing *heated* blood.

Occurrence.—Present in sputum, especially in the early stages. It would appear from the evidence available that *B. pertussis* is the causal agent, but this has not been fully proved.

Diagnosis.—A convenient method of isolating the organism for diagnostic purposes is to hold for fifteen seconds a plate of the Bordet-Gengou medium four inches in front of the mouth of the patient while coughing. The plate is thus inoculated directly with the droplets of sputum. After two days' incubation the "pearly" colonies of *B. pertussis* can frequently be recognised without difficulty, and identified by microscopic and cultural methods.

BACTERIUM PNEUMOSINTES (*Psalister pneumosintes*)

This organism was first isolated from the nasopharynx of cases of epidemic influenza.

Morphology and Staining.—A minute cocco-bacillary organism measuring 0.15 – 0.3μ in length. After prolonged artificial culture, its length may attain 0.5 – 1μ . The organisms are usually found singly, but may be in pairs, and even in short chains of three or four individuals. *Bacterium pneumosintes* is stained best with well-ripened polychrome methylene blue, when the organisms appear deep purple in colour. It is Gram-negative.

In young cultures a certain proportion of the organisms are small enough to pass Berkefeld V and N filters.

Culture.—Obligatory anaerobe. Optimum temperature— 37° C. Grows well in Smith-Noguchi medium (p. 118), forming a haze in the medium around the fragment of kidney in three to four days. On anaerobic blood-agar plates minute clear transparent colonies are formed after about six days' incubation.

Experimental Inoculation.—It has been stated that young cultures inoculated intratracheally in rabbits

produce slight fever, and leucopenia due to a diminution of mononuclear cells. It is not fatal for these animals.

Occurrence.—Was originally found in the nasal washings of epidemic influenza in the first thirty-six hours of the disease. Similar organisms have been observed in the throats of healthy persons, and in other pathological conditions of the respiratory passages.

It is doubtful whether this type of organism has any pathological relationships.

BACILLUS OF DUCREY (*Haemophilus ducreyii*)

Associated with "Chancroid" or "Soft Sore."

Morphology.—A rod-shaped organism 1.5μ by 0.4μ ; occurs in pairs and chains; present in the exudate from the sore, in the tissue lesion and in the secondary buboes; it is non-motile and non-sporing.

Staining.—Gram-negative.

Culture.—This organism has proved difficult to cultivate artificially, and appears to be a strict parasite. It has been found that primary cultures can be obtained, however, from the sore by inoculating directly tubes containing coagulated rabbit blood. These are prepared by distributing fresh rabbit blood, withdrawn from the ear vein or by cardiac puncture, in amounts of 1–2 c.c. in small test-tubes. When the blood has clotted, the tubes are heated at 55° C. for five minutes. After growth in these, the organism can be isolated on blood-agar plates. This method has been used for diagnostic purposes.

Pure cultures can also be obtained by puncturing a bubo with a syringe, drawing up some of the pus, and with this inoculating tubes of coagulated blood.

Haverhillia multiformis (*Streptobacillus moniliformis*)

This organism was reported in France in cases diagnosed as erythema multiforme, and named *Streptobacillus moniliformis*; it was also described in America in a group of cases of multiple

arthritis associated with an erythematous eruption (Haverhill Fever—*Erythema arthriticum epidemicum*). It has been found associated with a disease of mice characterised by multiple arthritis often involving the joints of the feet and leading to swellings of the feet and legs. It has been noted that the organism may occur in the nasopharynx of rats.

The organism is a Gram-negative, pleomorphic bacterium, occurring as short rod-shaped forms ($1-3\mu \times 0.3-0.4\mu$) or as elongated filaments which may show characteristic fusiform enlargements. Growth can be obtained in the presence of blood, serum or ascitic fluid, and a high proportion of these is required in the medium. Löffler's serum medium serves well for cultivation. The colonies are small (1 mm.). Viability in culture is feeble and cultures die out in two to four days.

Mice are susceptible to experimental inoculation and develop either a rapidly fatal general infection without focal lesions or a more slowly progressive disease with swelling of the feet and multiple inflammatory lesions of joints.

In the human infection the organism has been isolated by blood culture and from joint fluid in cases with arthritis.

CHAPTER XVII

BACILLUS TETANI; THE ANAEROBIC BACILLI OF INFECTED WOUNDS; BACILLUS BOTULINUS; BACIL- LUS CHAUVOEI

BACILLUS TETANI (*Clostridium tetani*)

THE causative organism of tetanus.

Morphology.—Straight, slender, rod-shaped organism $2-5\mu$ by 0.4μ , with rounded ends; shorter forms and longer filaments are also noted; motile, due to numerous long wavy flagella peritrichate in arrangement, but movement is not markedly active. Characteristic spores are developed—spherical, two to three times the diameter of the bacillus, and situated terminally, they produce the “drum-stick” appearance which is a striking morphological feature of the organism.

Staining.—Gram-positive, but different individuals may show variation in the reaction to Gram’s stain; by the ordinary staining methods, only the periphery of the spore is stained.

Culture.—An obligatory anaerobe, but growths can be obtained in the presence of minimal traces of oxygen; temperature range— $14^{\circ}-43^{\circ}\text{C.}$, optimum— 37°C. ; grows on ordinary nutrient media; glucose is often added to culture media as a reducing agent; the cooked-meat medium described on p. 121 and media prepared from a peptic digest of blood (p. 387) are suitable for the growth of the tetanus bacillus. For methods of anaerobic cultivation see p. 136.

Agar stab culture—no growth occurs on the surface;

a white line of growth appears along the needle track, but stops short of the surface, and lateral spikes, which are longest in the deeper part of the tube, develop from the central growth.

Colonies on agar are flat and delicate, with long branching projections. After forty-eight to seventy-two hours' incubation the central part of the colony, which rarely grows more than 1 mm. in diameter, becomes slightly raised above the surface of the medium and has a "ground-glass" appearance. The edge of the colony shows a feathery appearance, and growth tends to spread over the surface of the medium by means of the radial projections from the colonies.

Milk—no coagulation.

Gelatin—is slowly liquefied.

Coagulated serum—is rendered more transparent and softened but is not liquefied.

In bouillon—produces a uniform turbidity.

Cooked-meat medium—gas production but no noticeable digestion or blackening of meat.

No carbohydrates are fermented.

Cultures have a slight putrefactive odour.

Viability of Spores.—The spores possess a high resistance to adverse agents. They withstand boiling water for forty to sixty minutes, and even longer. They resist dry heat at 150° C. for one hour, 5 per cent. phenol and 1:1000 perchloride of mercury for considerable periods (two weeks or more). Iodine in watery solution and hydrogen peroxide (10 volumes) kill them within two hours.

The toxin is an exotoxin and can be prepared artificially by growing the organism in bouillon and filtering through an earthenware filter after ten to fourteen days' growth. There are two constituents—*tetanospasmin*, which acts on the nervous system, and also *tetanolysin*, which causes lysis of red blood corpuscles.

The tetanus toxin is an extremely powerful poison. When injected into guinea-pigs or mice, the animals die in twelve to eighteen hours with the typical signs of tetanus. The toxin reaches the central nervous

system by passing along the motor nerves, being absorbed probably by the motor end-plates and spreading up along the axis cylinders. It acts as an irritant to the motor cells in the anterior horn of the spinal cord and also interferes with the normal inhibition of motor impulses exercised by the upper motor neuron over the lower, producing increased muscular tonus and tonic spasms.

An antitoxic serum can be produced by immunising horses with toxin. This serum is of great value in the prophylaxis of tetanus. Its use as a curative agent after the development of tetanus is less reliable than, and not nearly so effective as, the corresponding antitoxin treatment in diphtheria.

Tetanus antitoxin is now standardised in Great Britain and Germany in terms of the "International Unit," which may be defined as five times the quantity of antitoxic serum required to preserve the life of a 350-gram guinea-pig for ninety-six hours when mixed with 100 M.L.D. of toxin and injected subcutaneously. This unit is half the value of the U.S.A. unit. The method of determining the potency of a serum is similar to that used in testing diphtheria antitoxic sera. For prophylactic purposes 3000 I.U.'s should be given subcutaneously and repeated weekly until the wound has healed. In the treatment of a case the antitoxin is administered both intraspinally (40,000 to 60,000 I.U.'s) and intravenously (20,000 I.U.'s), and the administration is repeated if necessary.

Occurrence.—The spores are widespread in nature, but are specially prevalent in manure, and manured soil. Tetanus bacilli may occur naturally in the intestine of certain animals—*e.g.* horses, cattle, sheep—and have been noted occasionally in the human intestinal contents. It is doubtful whether this organism flourishes as a saprophyte in nature, or is derived entirely from an animal source.

Tetanus is usually the result of a wound contaminated with tetanus spores. The infection remains strictly localised, and the tetanic condition is due to the diffusible toxin absorbed into the central nervous system.

Certain conditions favour the germination of the spores and the propagation of the organisms in the tissues—*e.g.* infection by other organisms (*B. welchii*, pyogenic cocci, etc.), foreign bodies such as pieces of clothing carried into the wound, necrotic tissue, and effused blood. It has been shown that the spores germinate only under a reduced oxygen tension.

B. tetani infection may occur in the uterus, as in cases of septic abortion, and also in the umbilical wound of new-born infants. Cases of post-operative tetanus have been recorded due to imperfectly sterilised catgut.

DIAGNOSIS

- (1) Films may be made from the wound pus or exudate and stained by Gram's method, but the appearance of "drum-stick"-like bacilli is not conclusive evidence of the presence of *B. tetani*, as other organisms having round terminal spores, which are practically identical in morphological characters, may be present. Moreover it is often difficult or impossible to detect the tetanus bacilli in wounds by microscopic examination.
- (2) The most reliable method for diagnostic purposes is to produce tetanus in white mice by subcutaneous injection of a filtrate of an anaerobic fluid culture from the wound.
- (3) Cultures are made from the infected wound or other material, and in isolating the organism advantage is taken of the tendency of *B. tetani* colonies to spread and extend beyond the growth of other bacteria.

In Fildes' method the material is incubated anaerobically in 5 per cent. peptic-blood-broth¹

¹ A mixture of 150 c.c. saline solution, 6 c.c. pure HCl, 50 c.c. defibrinated sheep blood and 1 gram of pepsin (B.P. granulated) is heated at 55°C. in a stoppered bottle for two to twenty-four hours. There is then added sufficient 20 per cent. NaOH solution (usually about 12 c.c.) until the

for two to four days at 37° C. The culture is then heated at 80° C. for half-an-hour to kill spreading non-sporing organisms such as *B. proteus*. The condensation water of a peptic-blood-agar slope is inoculated from the heated culture, and incubated at 37° C. under anaerobic conditions. After twenty-four to forty-eight hours the edge of the culture is examined with a low-power binocular microscope or hand-lens, when a growth of tetanus bacilli is seen as a mass of very fine filaments. Subcultures from the marginal growth usually yield pure cultures of *B. tetani*. (It is advantageous to keep the peptic-blood-agar tubes until the surface of the medium is dry at the top.)

THE ANAEROBIC BACILLI OF INFECTED WOUNDS

These organisms, belonging to the genus *Clostridium*, are associated with rapidly spreading inflammatory oedema, necrosis and gangrene of the tissues, and gas production, occurring as a complication of wound infection. They are all sporing organisms and their source is mainly animal excreta. They were responsible for the so-called gas-gangrene which was so prevalent among the armies in Europe during the War. The heavily manured soil of the cultivated fields of France contained abundant spores capable of remaining viable for long periods of time, and the infection resulted from the contamination of wounds with soil.

sample of the mixture diluted with water gives a permanganate-red colour with cresol-red indicator. Pure HCl is now added drop by drop until a sample of the mixture shows almost no change of colour with cresol-red, but a definite red tint with phenol-red. It is important to avoid excess of acid. Chloroform, 0.25 per cent., is added and the mixture shaken vigorously. This peptic digest of blood keeps well for months. For use it is heated to 55° C. for thirty minutes to remove the chloroform, and added to broth or agar in the proportion of 2 to 5 per cent.

In nature, these organisms play an essential part in the process of putrefaction.

They have been broadly classified into two types : (1) *saccharolytic*, (2) *proteolytic*.

(1) The saccharolytic organisms are characterised by their rapid and vigorous growth in carbohydrate media with the production of acid and abundant gas. If grown in a medium containing both carbohydrate and protein—*e.g.* cooked-meat medium (*vide* p. 121)—there is rapid production of acid and gas, but *no digestion* of the meat. The cultures have a slightly sour smell, and the meat is turned pink by the acid.

(2) The proteolytic type is characterised by the digestion of protein. In the cooked-meat medium the proteolytic ferments evolved by these organisms decompose and blacken the meat with the formation of foul-smelling sulphur compounds. The protein is also broken up into amino-acids, and small white feathery masses of tyrosine crystals are frequently seen in cultures.

The more important organisms exemplifying these types are the following :—

<i>Saccharolytic</i>	<i>Proteolytic</i>
(1) <i>B. welchii</i>	(1) <i>B. sporogenes</i>
(2) <i>Vibrio septique</i>	(2) <i>B. histolyticus</i>
(3) <i>B. tertius</i>	<i>B. tectani</i> (<i>vide</i>
(4) <i>B. fallax</i>	p. 385) is
(5) <i>B. oedematiens</i> (<i>B. novyi</i>)	slightly proteolytic

There is, however, no hard and fast line of demarcation between the two groups. Thus, strains of *B. welchii* produce small amounts of amino-acid, and *B. sporogenes*, though essentially proteolytic, has saccharolytic properties.

For further detailed information on the anaerobes of infected wounds, the *Medical Research Committee's Special Report*, No. 39, 1919, should be consulted. A useful short Table (quoted from p. 47 of the Report) is given on p. 390, showing the general classification of these organisms.

TABLE SHOWING CLASSIFICATION OF SPORING ANAEROBES

	Both proteolytic and saccharolytic properties	Slight proteolytic but no saccharolytic properties	Saccharolytic but no proteolytic properties	Neither saccharolytic nor proteolytic properties
	Proteolytic properties predominating Coagulated serum and gelatin are liquefied	Saccharolytic properties predominating Serum not liquefied; gelatin liquefied		
Central or sub-terminal spore	<i>B. sporogenes</i> <i>B. parasporogenes</i> <i>B. histolyticus</i> <i>B. aerofetidus</i> <i>B. bifermentans</i>	<i>B. welchii</i> <i>Vibrio septique</i> <i>B. chaurvici</i> <i>B. oedematis</i> (<i>B. noryi</i>)	<i>B. fallax</i> <i>B. multifermens</i> <i>B. tendus</i>	
Oval terminal spore			<i>B. tertius</i>	<i>B. cochlearius</i>
Spherical terminal spore		<i>B. tetani</i>	<i>B. tetanomorphus</i> <i>B. sphenoides</i>	

This Table is quoted by permission of the Controller of H.M. Stationery Office, from the *Medical Research Committee's Special Report*, No. 39.

It must be emphasised that the separation and cultivation of these anaerobes is much more difficult than in the case of the aerobes. The ordinary simple laboratory media are ill-adapted for the purpose, and it is necessary to use media rich in carbohydrate or protein, according to the organism studied. Moreover, the reaction of the medium is of paramount importance, as it is probably only within a certain range of reaction that the bacterial ferments can enable the organism to utilise the food material present.

Having obtained a suitable medium, further difficulties are met with in the separation of the organisms. In wounds there is practically always a mixed infection, so that the isolation of the various bacteria must be undertaken. Simple plating alone is not sufficient as in the case of the aerobes, but alternate growths on plates and in fluid media over several generations are necessary before a pure culture is obtained. Much of the confusion in the early literature has been due to the failure to separate mixed cultures, and according as the medium contained carbohydrate or protein, so the saccharolytic or proteolytic organisms in the mixtures predominated.

For infection by these anaerobes to take place, *dead tissue or blood clot must be present.* The organisms cannot attack living tissue directly. During the War, infection occurred where there were deep lacerated wounds caused by irregularly shaped pieces of shell, and into which muddy clothing and particles of earth were carried. Spores were thus introduced under most favourable conditions for the development of the organism.

In the contaminated wound the saccharolytic organisms are the first to grow. Toxic products of growth gain access to the muscle fibre where it is torn, and the toxic fluid causes death of the fibre by passing between it and the sarcolemma sheath. In the dead muscle fibre the saccharolytic organisms (usually *B. welchii*) rapidly multiply, fermenting the muscle sugar and forming abundant gas. The death of the muscle is assisted by the oedema and gas formation,

which tend to cut off the blood supply. The process spreads along the injured fibres throughout the length of the muscle. The proteolytic organisms (usually *B. sporogenes*) follow later, digesting the dead muscle and causing blackening and the foul odour.

BACILLUS WELCHII (B. PERFRINGENS, B. AEROGENES
CAPSULATUS, *Clostridium welchii*)

The most frequent organism in, and the commonest cause of, gas gangrene.

Morphology.—A relatively large Gram-positive bacillus, about $4-6\mu$ by 1μ , with square or rounded ends, occurring singly or in pairs, and often capsulated when seen in the tissues. In sugar media the bacilli are shorter, while in protein media they tend to become filamentous. The bacilli are non-motile. Spores are formed, but only in the absence of fermentable carbohydrates. They are oval, central or subterminal.

Culture.—Anacrobe. Optimum temperature about 37°C . Grows best on carbohydrate-containing media—*e.g.* glucose-agar.

Surface colonies—round, smooth, regular, opaque discs. (Another type of colony is also observed, with an opaque centre and a transparent border which is radially striated.)

In milk—acid, clot and gas production result; the gas breaks up the clot, producing the characteristic “stormy clot” reaction; the culture has a sour, butyric-acid odour.

Gelatin is liquefied.

Solidified serum—no liquefaction occurs.

B. welchii is actively saccharolytic and ferments with gas production, glucose, lactose, saccharose, maltose, starch, and, in the case of some strains, glycerol and inulin.

In the cooked-meat medium (*vide* p. 121)—the meat is reddened and no digestion occurs.

Filtrates of cultures are strongly haemolytic, and

a lethal exotoxin can be demonstrated by intravenous injection of mice.

An antitoxin is obtainable by immunising an animal—*e.g.* a horse—with the toxin.

Occurrence.—Apart from its pathological relationships, *B. welchii* occurs normally in the large intestine of man and animals. It may invade the blood ante-mortem, and multiplying in internal organs after death produces the small gas cavities sometimes noted (*e.g.* in the liver) at post-mortem examinations. Apart from wound infections it may occur in uterine infections (*e.g.* septic abortion) and in infections of the intestinal tract, gall bladder and the urinary system.

In certain conditions the numbers present in the bowel are greatly increased—*e.g.* pernicious anaemia, intestinal obstruction. In pernicious anaemia the associated achlorhydria is probably a factor which allows this organism to flourish, and in this condition *B. welchii* may be found even in the duodenum and stomach.

B. welchii may also occur in gas-oedema of the muscles in cattle and sheep.

Animal Inoculation.—The virulence varies greatly with different strains. Some are markedly pathogenic to guinea-pigs by subcutaneous injection of culture and the animal may die within twenty-four hours. At autopsy a spreading inflammatory oedema with gas production is noted in the subcutaneous tissue, and necrosis in the underlying muscles, which are sodden, friable and pink. Organisms from cultures washed with saline solution to free them from toxin and other soluble products are non-pathogenic. Apparently the products of growth of the bacillus increase its aggressiveness.

Pigeons are the most susceptible of laboratory animals to experimental inoculation.

Lamb Dysentery.—This condition has been shown to be due to a sporing anaerobic bacillus with close relationships to *B. welchii*. Its pathogenicity depends on exotoxins for which

an antitoxic serum can be obtained. This serum has been utilised with great success in the prophylaxis of the disease.

Another organism resembling *B. welchii*, and named *B. paludis*, has been found associated with a disease of sheep in Kent known as "struck," the condition being essentially a toxæmia due to absorption of the bacterial toxins from the small intestine. A similar disease has been reported in Australia, and designated "infectious entero-toxæmia"; the associated organism, also related to *B. welchii*, has been designated *B. ovitoxicus*. A disease affecting lambs in Australia and in this country, often called "pulpy kidney disease," is probably of similar nature and aetiology to infectious entero-toxæmia.

Five different toxins, distinguishable by neutralisation tests with antitoxic sera, have been demonstrated among the organisms of the *B. welchii* group, and individual strains may produce more than one of these. The types of *B. welchii* referred to above tend to differ as regards their production of the several toxins.

VIBRION SEPTIQUE (*Clostridium oedematis-maligni*)

Morphology and Staining.—Moderately large bacillus, with rounded ends, about $3-10\mu$ by $0.6-1\mu$. Motile, due to flagella (peritrichate). Tends to grow also in the form of long curved filaments. In the tissues it develops into large, swollen, Gram-positive, lemon-shaped forms, which are designated "citron bodies." Spores are readily formed and are oval, central, or subterminal, and "bulging." *V. septique* stains Gram-positively as a rule, but degenerate forms are Gram-negative.

Culture.—Anaerobe. Optimum temperature, 37°C . Capable of growing on ordinary media. Glucose promotes growth.

Agar stab—a white line of growth with short lateral processes.

Surface colonies—irregular transparent droplet-like colonies, later becoming greyish and opaque, with projecting radiations like those of *B. tetani*.

Milk—acid is formed, and the milk is slowly clotted.

Gelatin is liquefied.

Solidified serum—no liquefaction.

Cooked-meat medium--meat is reddened but not digested.

Various sugars are fermented.

Exotoxin can be demonstrated in cultures.

Animal Inoculation.—Subcutaneous injection of cultures in laboratory animals produces a spreading inflammatory oedema, with slight gas formation in the tissue. The organisms invade the blood and the animal dies within a day or two. Smears from the liver show long filamentous forms and also “citron bodies.”

V. septicæ is responsible for *Braxy* in sheep and some cases of *Black-leg* in cattle (*vide infra*).

A vaccine consisting of formolised culture of this organism has been used with success in the prevention of braxy.

BACILLUS SPOROGENES (*Clostridium sporogenes*)

An anaerobic motile bacillus, with peritrichate flagella, and oval, usually subterminal, spores. It is about the same size as *B. welchii*, but more slender. It is typically Gram-positive in young cultures, but Gram-negative forms are frequent in older cultures.

A stab culture shows a growth like that of *B. tetani*, with lateral radiations or spikes. Surface colonies are yellowish and feathery. Cultures have an exceedingly putrid odour. In milk the casein is precipitated and digested. In cooked-meat medium the meat is blackened and digested. Solidified serum is liquefied. Glucose and maltose are fermented, with gas production.

The organism decomposes protein, producing amino acids, ammonia, sulphuretted hydrogen, etc.

It is non-pathogenic to laboratory animals.

Bacillus novyi (*B. oedematiens*, *Clostridium oedematiens*).—This organism resembles *B. welchii* in morphology but is somewhat larger and more pleomorphic. It possesses peritrichate flagella but its motility is not active. The spores are sub-terminal as a rule. Surface colonies are flat and tend to fuse, forming a spreading film of growth. Deep colonies are small, irregular, “snow-flake”-like balls of growth. In milk, acid and

clot are slowly formed. Gelatin is liquefied but coagulated serum is not digested. The organism is actively saccharolytic and various sugars are fermented. In cooked-meat medium the meat is reddened but not digested. A highly potent exotoxin is produced and this organism has been found specially associated with a "toxic" form of gas-gangrene.

B. novyi has also been found associated with a braxy-like disease in sheep.

Bacillus fallax (*Clostridium fallax*) resembles *B. welchii* in some respects, and has sometimes been mistaken for it (hence the name "fallax"). It is, however, shorter and more slender, and is motile, possessing peritrichate flagella. The spores are subterminal. In milk the organism produces clotting and gas formation, but these changes take place slowly (as compared with *B. welchii*). It does not liquefy either gelatin or coagulated serum, and is non-proteolytic. It possesses restricted saccharolytic properties. An exotoxin is formed, and the organism is pathogenic on experimental inoculation in animals.

Bacillus tertius (*Clostridium tertium*).—In morphology this organism tends to be long and slender. It is weakly motile. The spores are terminal, and when fully developed oval in shape. Neither gelatin nor coagulated serum is liquefied, but the organism shows active saccharolytic properties. In milk a soft coagulum is formed and a certain amount of gas. Meat is reddened but not digested. Its pathogenicity is doubtful, but when present in wounds it may give rise to gas production.

Bacillus histolyticus (*Clostridium histolyticum*) resembles *B. sporogenes* and is actively proteolytic. In meat medium digestion occurs with the formation of white crystalline masses consisting probably of tyrosine; the culture does not emit a putrid odour. When cultures are injected into animals, *in vivo* digestion of the tissues results.

DIAGNOSIS

The presence of *B. welchii* may be shown by inoculating melted glucose agar in tubes at 50°C. and allowing to solidify. After twelve hours' incubation the medium is split up by abundant gas, and the cotton-wool stopper and even some of the medium may sometimes be forced out of the tube. Microscopical examination shows the typical Gram-positive bacilli. Inoculation into milk, which is incubated under anaerobic conditions, shows the typical "stormy clot" in six to twelve hours.

B. welchii can be isolated in the pure condition by sub-culturing in a tube of cooked-meat medium, and after six hours' incubation, sub-inoculating a similar tube. This second culture is plated on agar smeared with alkaline-egg medium, and grown anaerobically for twelve hours. Colonies are then transferred to meat medium and two successive inoculations are made at six hours' intervals. Altogether the culture must be passed alternately through two tubes of cooked-meat medium and one plate, nine or ten times, before a pure culture can be ensured.

B. sporogenes can be detected by inoculating the material into cooked-meat medium. After two days' incubation, blackening and digestion of the medium with a foul odour is presumptive evidence of the presence of this organism. On microscopic examination, the characteristic bacilli with sub-terminal spores, or "racquet" forms are seen.

Pure cultures may be obtained by alternate growths for three days in meat medium and three days' on agar plates (anaerobic) smeared with alkaline-egg medium.

BACILLUS BOTULINUS (*Clostridium botulinum*)

The organism of "Botulism," a fatal form of food-poisoning characterised by pronounced toxic effects mainly on the parasympathetic system—*e.g.* oculomotor paralysis, pharyngeal paralysis, aphonia, etc.

Morphology and Staining. A sporing bacillus with rounded ends, about $4-6\mu$ by $0.9-1.2\mu$, occurring singly and in pairs. Spores are oval, subterminal and slightly "bulging." The bacilli are motile, with peritrichate flagella, and stain Gram-positively unless degenerate.

Culture.—Anaerobe. The optimum temperature has been variously stated; earlier workers found growth occurred best at $20^{\circ}-30^{\circ}\text{C.}$; more recent observations indicate that the optimum is about 35°C. Grows on ordinary media; cooked-meat medium yields abundant growths.

Agar culture—a white line of growth, stopping short of the surface, with short lateral spikes or radiations; gas production is marked, especially in glucose agar.

Surface colonies—large, greyish, irregular, semi-transparent, with a central “nucleus” and a reticular border. Cultures emit a rancid, butyric-acid odour.

Gelatin is liquefied.

Solidified serum—slow liquefaction generally occurs.

Milk—unchanged or curdled, or in some cases slightly acidified without clotting or digestion.

Ferments glucose and maltose.

Cooked meat—action on meat is somewhat variable; most strains digest and darken the meat.

In culture media and in contaminated foods, *B. botulinus* produces a powerful exotoxin which is responsible for the pathogenic effects in the disease. This toxin is destroyed when exposed to a temperature of 90° C. for forty minutes.

Two types of the bacillus, designated A and B, have been recognised, producing toxins which are immunologically different.

Type A toxin is pathogenic for fowls, whereas type B is not. Antitoxin produced from toxin A does not neutralise toxin B, and vice versa.

Type A is also specially characterised by its fermentation of salicin.

Strains have also been described differing from types A and B—e.g. type C (*B. parabotulinus*). This variety does not digest meat (cp. A and B) and generally fails to liquefy coagulated serum. Glycerol is not fermented, though this substance is acted on by types A and B.

The condition of “*Limber-neck*” in fowls is generally regarded as a form of botulism, types A and C being the responsible organisms.

Other conditions in animals ascribed to botulism are: *Forage-poisoning* in horses and cattle (type B) and “*Lamziekte*” of cattle in South Africa (*B. parabotulinus*).

Occurrence.—Botulism has been found to originate from a considerable variety of preserved foods—e.g. ham, sausage, canned meats and vegetables, etc.

In the human subject botulism is due not to the formation of toxin by the organism in the intestine, but to the absorption from the stomach and upper

duodenum of *toxin pre-formed by the bacillus growing in the food*.

Canned foods responsible for botulism frequently exhibit signs of spoilage.

B. botulinus is a saprophytic organism and is widely distributed. Its natural habitat is soil, even virgin and forest soil. It may be found in vegetables, fruits, leaves, mouldy hay, ensilage and animal manure.

The spores of *B. botulinus* withstand moist heat at 100° C. for several hours. They are destroyed at 121° C. (moist heat) within twenty-two minutes. Insufficient heating in the process of canning foods is an important factor in the causation of this form of poisoning.

In cases of botulism the bacillus may be demonstrated in the stomach contents and faeces, and post-mortem in the intestinal contents and in the liver and spleen.

It can also be isolated from the food responsible for the outbreak.

Animal Inoculation.—Laboratory animals are susceptible to experimental inoculation and feeding with cultures. The resulting condition resembles an "acute bulbar paralysis" like the human disease; at autopsy marked congestion of the internal organs, extensive thrombosis and haemorrhages are noted.

Serum antitoxin can be prepared by immunising animals with toxin preparations, and is used therapeutically. A bi-valent serum containing antitoxins to both the A and B types of toxin should be employed.

DIAGNOSIS

As the condition of botulism is essentially a food intoxication, the suspected food calls for investigation.

It is macerated in sterile salt solution, heated at 60° C. for half-an-hour to eliminate non-sporing bacteria, and then cultures are made under anaerobic conditions—*e.g.* in cooked-meat medium. A culture-filtrate is obtained and its toxicity tested by injection of guinea-pigs or mice. For control purposes the filtrate is also injected into animals along with

antitoxin. *B. botulinus* can be isolated in pure culture by appropriate plating methods, and identified by its biological characters and its toxicity.

An extract should also be made from the food, sterilised by filtration through a porcelain filter, and injected subcutaneously into guinea-pigs.

BACILLUS CHAUVOEI (*Clostridium chauvoei*)

The causative organism of quarter evil (blackleg, or symptomatic anthrax) in cattle, a disease characterised by a swollen and emphysematous condition of the subcutaneous tissues and muscles. The infection may possibly originate from a surface wound, and frequently affects the fore- and hind-quarters, which become dark or almost black in colour.

Morphology.—Resembles closely the *Vibrio septique* and is $3-5\mu$ in length and $0.5-0.6\mu$ broad. Individual organisms tend to occur singly or in pairs and not in long filaments. They are actively motile, having numerous lateral flagella. Spores are usually central or subterminal in position, elliptical in shape, and are broader than the bacillus.

Staining.—Gram-positive in young cultures but older forms may be Gram-negative. Stains readily with ordinary dyes.

Culture.—Anaerobe; optimum temperature 37°C ., but grows at room temperature. Grows on ordinary medium but an albuminous or tissue medium containing glucose is preferable.

Colonies on agar—greyish rounded discs with radiating filamentous processes.

Stab culture in glucose agar—growth only commences some distance below the surface: along the puncture a whitish line appears with lateral projections, the growth being luxuriant.

Milk may remain unchanged or show acid formation.

Gelatin is liquefied.

Coagulated serum is not liquefied.

Cooked-meat medium—meat is reddened and is not digested.

Ferments glucose, lactose, saccharose, but not mannite, salicin or inulin.

A powerful toxin is obtained in glucose-broth cultures if calcium carbonate be added to neutralise the acid produced.

Occurrence and Pathogenicity.—The disease occurs in sheep and cattle. The organism is pathogenic for guinea-pigs and mice, these animals dying twenty-four to thirty-six hours after inoculation. At autopsy there is an extensive blood-stained oedema round the site of inoculation and the muscles present a dark red or black appearance, while there is a considerable amount of gas produced; the bacillus is present in the heart

blood in pure culture. It has been supposed that the natural disease results from infection of a wound by the spores which may be present in the soil of infected pastures, but there is evidence that infection may also take place through the alimentary tract.

Immunity.—Immunity to the disease was at one time produced by the inoculation of a dried powder of the muscles of animals dead of the natural disease, the powder being subjected to a suitable temperature to ensure the attenuation of the virulence of the contained spores. A mixture of virus, as above, with antitoxin was also used to protect susceptible animals. More recently a formalised culture of *B. chauvoei* in broth has been applied with successful results.

V. septicæ may also be responsible for a condition similar clinically to "black-leg."

In the *differentiation of V. septicæ and B. chauvoei*, stress is laid on the morphological elements seen in infected guinea-pigs: *B. chauvoei* exhibits "citron" and club-shaped forms, but no elongated filaments are observed on the peritoneal surface of the liver as in the case of *V. septicæ*. *B. chauvoei* ferments saccharose but not salicin; *V. septicæ* ferments salicin but not saccharose.

CHAPTER XVIII

ACTINOMYCES AND OTHER PATHOGENIC STREPTOTHRICES; LEPTOTHRIX; BACILLUS RHUSIOPATHIAE (SWINE ERYSIPELAS); BACILLUS FUSIFORMIS; BACILLUS NECROPHORUS

ACTINOMYCES

THE causative organism of actinomycosis in animals and man.

This organism belongs to the genus *Streptothrix* of the Higher Bacteria (*vide* p. 5), and may be designated "*Streptothrix actinomyces*."

Morphology.—The organism grows as a mycelium or felted mass of branching filaments which are comparatively slender (0.7μ thick) and are enclosed in a sheath containing pigment granules. In the centre of this mycelial colony the filaments interlace irregularly, but at the periphery there is a tendency to radial arrangement. The organisms show true dichotomous branching. The mycelium is embedded in a ground-work or matrix. In old growths the filaments become matted together into a structureless mass. They also show fragmentation into bacillary and coccal forms. The formation of conidia has not been demonstrated. In culture the typical mycelium may not be so obvious as in the tissues, and the growth may be composed largely of shorter bacillary forms resembling a diphtheroid bacillus; among these, however, are seen some longer branching filaments which reveal the true character of the organism. Growing in the

tissues (especially in animals) the actinomyces colony develops pyriform or club-shaped structures at the periphery, supposed to result from the swelling of the sheath at the extremities of peripheral radial filaments. These "clubs" lie radially with their wide end outwards and as seen in tissue sections form a complete ring round the colony. In animal lesions the clubs may constitute the only morphological feature of the older colonies, owing to the degeneration of the filaments, which become fused into a structureless mass in the centre of the colony. The clubs are regarded as a defensive mechanism on the part of the organism against the tissues of the host. In human lesions, club formation is comparatively rare.

Staining.—The filaments are Gram-positive. The clubs usually stain Gram-negatively, but are acid-fast, and can be stained differentially by the Ziehl-Neelsen method, 1 per cent. being substituted for 20 per cent. sulphuric acid.

Culture.—Two main cultural types have been recognised :

- (1) The aerobic type, exemplified by Boström's classical strain; it has been suggested that this type is not pathogenic and that its occurrence in lesions represents a secondary contamination; similar organisms are common saprophytes on grain and grasses.
- (2) The "anaerobic" type of Israel and Wolff, which is the prevalent form in animal and human lesions.

Boström's Aerobic Type (Actinomyces hominis).—Temperature range—20°–40° C., optimum—37° C. Grows on ordinary media, but the presence of serum or glycerol encourages growth.

On agar—grows slowly; colonies begin to appear after four or five days; when well developed, they stand out on the surface of the medium as discrete, rounded, yellow, transparent knobs, often likened to "amber drops"; they are firmly adherent to the

medium; older colonies become umbilicated and assume a dry appearance.

In gelatin—slow liquefaction occurs.

No pathogenic effects have been demonstrated by experimental inoculation.

Anaerobic Type (Actinomyces bovis).—The optimum temperature is 37° C., and growth does not occur at temperatures much below the optimum.

On agar (under anaerobic conditions) colonies are raised, greyish-white, opaque discs which tend to show a rosette form and are firmly adherent to the medium.

A shake culture in a tube of agar presents a characteristic distribution of the colonies, which are most numerous in a zone from 10–20 mm. below the surface—i.e. where there is only a trace of free oxygen present.

If the organism is grown in the form of a stab culture in agar, growth is also at an optimum in a similar zone. Though for all practical purposes this organism is anaerobic, it shows a micro-aerophilic character, a minute trace of oxygen being more favourable to growth than complete anaerobiosis.

By experimental inoculation of cattle and laboratory animals (e.g. rabbits, guinea-pigs) circumscribed nodular or “tumour-like” granulomatous lesions have been produced, in which colonies of the organism are demonstrable.

Occurrence.—Actinomycosis is an infective granuloma, occurring mainly in cattle, sheep and pigs, and occasionally in man. In human cases the lesions usually show a suppurative tendency, and the pus contains colonies of the parasite in the form of small round granules about the size of a pin-head, which are occasionally of a bright yellow colour (like grains of sulphur). These granules can be recognised by the naked eye if the pus is examined in the form of a thin layer on a slide. The commonest avenue of infection in man is through the mucosa of the mouth or throat. The mode of infection is often doubtful, and it may be impossible to trace the source. The initial infectivity

of the organism is probably weak, and invasion of the tissues may occur only in the presence of some additional factor. Primary foci have been noted in animals, and occasionally in man, around fragments of grain embedded in the mucous membrane of the mouth. It has been thought that grain might be a primary source of the infection, but it is more likely in these cases that the parasite has gained independent access to the body, and that the grain fragment facilitates the establishment of the organism in the tissue. The prevalent type of actinomyces (*vide supra*) is, in fact, a strict parasite, and incapable of a saprophytic existence on grain. Where the avenue of infection is by the mouth or throat, the primary lesions involve the soft tissues of the mouth and neck, the periosteum of the jaw, and even the vertebrae. In some cases the avenue of infection may be through the mucosa of the bowel (*e.g.* caecum), or the primary lesion may be in the lung. A certain amount of evidence has been adduced to suggest that the actinomyces may sometimes occur as a commensal in the mouth and the alimentary tract.

Metastatic lesions are also liable to occur (*e.g.* in the liver, brain, kidneys, or lung) by blood-stream spread.

The organism is found in tissue lesions, as in pus, in the form of compact colonies or granules which are visible even to the naked eye, and these present the microscopic appearances described above, varying according to the age of the individual colonies.

BACILLUS ACTINOMYCETEM COMITANS.—Besides the mycelial organism, a small Gram-negative coccobacillus (in morphology not unlike *B. melitensis*) is frequently present in large numbers in the actinomyces colony. This organism can be cultivated independently. Growth occurs under aerobic conditions, and consists of small colonies like those of a streptococcus and entirely different from the actinomyces. This organism has been regarded as a distinct species, and has been designated *B. actinomycetem comitans*, but its actual relationship to the actinomyces is unknown. Experimental inoculation does not produce any specific lesions.

DIAGNOSIS

If the pus from an actinomycotic lesion is spread out in a thin layer in a Petri dish or on a microscope slide, the characteristic colonics or granules can be recognised with the naked eye. For microscopic examination the granules in a drop of pus are "crushed" between two slides. In this way films can be prepared and then stained by Gram's method.

The granules can easily be separated by shaking up the pus with water in a test-tube, allowing them to sediment, and collecting them in a capillary pipette. They are then deposited on a slide and films made by crushing. Preparations obtained in this way are more satisfactory than those made directly from pus in which the granules may be relatively scanty.

Microscopic demonstration of Gram-positive branching filaments arranged in the form of a mycelium is generally sufficient for clinical diagnosis.

In tissue lesions the colonies can be recognised by preparing histological sections and staining by Gram's method, and, in the case of animal lesions, by the modified Ziehl-Neelsen method described above.

To cultivate the organism it is essential that actual granules should be used for inoculating the medium. For this purpose, the pus is mixed with sterile water, the granules are allowed to sediment, and then removed with a pipette; this is repeated two or three times so that the granules are thoroughly washed. This procedure is particularly necessary when there is mixed infection. Treatment of the granules with absolute alcohol before implanting them in medium facilitates the isolation of the organism by destroying the associated pyogenic cocci (*vide* p. 437). Two agar plates are inoculated with the separated granules. One is incubated aerobically, the other anaerobically.

ACTINOBACILLUS (*Actinobacillus lignieresii*)

This organism has been cultivated from a large proportion of cases of bovine "actinomycosis." In such cases no typically mycelial organism can be detected in the tissues and the

granules may be composed almost entirely of "club" formations. No mycelial organism can be cultivated, but on ordinary medium under aerobic conditions a small Gram-negative bacillus develops. This organism is about 1.5μ in length and is non-motile. The designation *Actinobacillus* has therefore been applied to it. In shake cultures in glucose agar elongated filamentous but unbranched forms are noted. The colonies are small, circular and translucent. Glucose, maltose, saccharose and certain other sugars are fermented. Inoculation of cultures into oxen and guinea-pigs reproduces lesions characteristic of actinomycosis with typical colonies or granules in the tissue.

OTHER PATHOGENIC STREPTOTHRICES

Apart from the typical actinomycosis, granulomatous and suppurative conditions occur in animals and man, due to infection by streptothrices, which differ biologically from the actinomyces. The following organisms may be taken as examples.

EPPINGER'S STREPTOTHRIX (*Actinomyces asteroides*)

Originally isolated from a brain abscess. The filaments are relatively broad (1μ in diameter), and very readily break up in culture into bacillary forms. They stain Gram-positively and are slightly acid-fast. This organism can be cultivated aerobically on ordinary medium as a friable, white, dry, wrinkled or nodular growth, which later becomes pigmented (yellow or pink).

ACTINOMYCES NOCARDII (*Actinomyces farcinicus*)

The organism of bovine "farcy," in which superficial lymph glands become swollen and ulcerate through the skin.

The organism shows mycelial formation, but in culture readily fragments into shorter bacillary and oval forms. It is Gram-positive and tends to be acid-fast. It grows aerobically at 37°C . on ordinary media, producing raised irregular greyish-white colonies after two to three weeks.

Guinea-pigs are susceptible to experimental inoculation and develop nodular or tubercle-like lesions. In bovines, subcutaneous injection leads to a localised abscess which breaks through the skin and produces a chronic ulcerated lesion. Rabbits are not susceptible to inoculation.

THE ORGANISMS OF MYCETOMA OR MADURA FOOT

Mycetoma is an infective granuloma localised usually to the tissues of the foot, and exhibiting no metastases. The condition occurs only in certain tropical and subtropical countries (*e.g.* India, some parts of Africa, etc.).

In the tissue lesion and pus, granules or colonies are noted as in the case of actinomycosis. These granules vary in colour; in some cases they are white or yellow ("pale variety"), in others black ("melanoid variety").

The *pale granules* usually represent colonies of a streptothrix—*e.g.* the *Streptothrix* (or *Actinomyces*) *madurae*.

Morphologically this organism resembles the actinomyces, but clubs are less frequently noted.

Cultural characters.—It is a strict aerobe. The optimum temperature is about 37° C. On nutrient agar the growth consists of circular raised colonies like those of the actinomyces, at first yellowish, later pinkish.

Other species of streptothrix have also been reported in cases of mycetoma.

The *black granules* in mycetoma represent hyphomycetes, which have been designated under the generic name of *Madurella*.

The colonies consist of a mycelium of broad, branching, septate hyphae 3–8 μ in breadth, and contain a considerable amount of black pigment.

This type of organism can be cultured on nutrient agar, and old cultures show the black pigmentation.

A number of different varieties have been described and this group has been regarded as related to the *Aspergillus*.

LEPTOTHRIX

An organism of this type (*vide* p. 5) is a common inhabitant of the mouth cavity, and may be detected in films made from the secretion or deposits of tartar between the teeth. It is designated *Leptothrix buccalis*.

Pathogenic properties have been claimed for it, but its invasive power is probably slight.

Leptothrix types have also been reported in suppurative lesions in the region of the mouth and throat.

BACILLUS RHUSIOPATHIAE (*Erysipelothrix rhusiopathiae*)

The causative organism of swine erysipelas.

Morphology.—Slender, Gram-positive, non-motile rod-shaped organism $1-2\mu$ by $0.2-0.4\mu$, occurring singly and in chains. In culture media, longer and filamentous forms are observed. True branching has been described.

Culture.—Growth occurs on ordinary media even at room temperature, though the optimum is about 37°C . The organism shows micro-aerophilic characters and in stab cultures on agar or gelatin does not grow on the surface. Surface growths may be obtained under anaerobic conditions. In gelatin stab culture a line of growth occurs along the needle track with lateral spikes or discs radiating from the central growth. Surface colonies on plates are exceedingly minute and dewdrop-like; they do not exceed 0.5 mm. in diameter when growing on agar.

Experimental Inoculation.—Mice, rats, rabbits and pigeons are susceptible to inoculation. Mice and pigeons are specially susceptible, and usually die of an acute septicaemia within four or five days after experimental inoculation. Subcutaneous injection in rabbits produces a spreading inflammation and oedema with a fatal result. Experimental inoculation (with cultures) in swine reproduces the disease as it occurs naturally.

Distribution.—The bacilli can be observed in the characteristic skin lesions, and in internal organs—*e.g.* lungs, spleen and kidney. In some cases there is a marked septicaemic condition and the organism is detectable in blood films, particularly in leucocytes. In the chronic form of the disease, in which a “verruccose endocarditis” occurs, the bacilli may be confined to the cardiac lesions.

For diagnostic purposes inoculation tests should be carried out in mice or pigeons.

Cases of human infection by this organism have been recorded.

B. rhusiopathiae may occur as a natural commensal in pigs, and has been isolated from the tonsils, intestine and faeces.

BACILLUS FUSIFORMIS (*Fusiformis dentium*)

This organism is referred to (p. 417) as a concomitant of spirochaetes of the “refringens” type, and is found in various necrotic inflammatory conditions along with

these spirochaetes—*e.g.* in Vincent's angina, ulcerative stomatitis, etc.—and occasionally in diphtheritic lesions of the throat.

Morphology.—It is a large, non-motile, fusiform bacillus, 5–14 μ by 1 μ .

Staining.—Gram-negative. The centre of the bacillus often stains less deeply than the poles, and a beaded or granular appearance may be noted.

Culture.—Anaerobe. It has been cultivated on a medium containing 1 part of blood to 3 of agar. The colonies are small white discs resembling a growth of streptococci.

BACILLUS NECROPHORUS (*Actinomyces necrophorus*)

This organism is responsible for diphtheritic and necrotic lesions in various animals—*e.g.* calf diphtheria, necrobacillosis in domesticated animals.

The organism appears in the form of elongated slender filaments varying in length and attaining sometimes to 80 or 100 μ . The filaments are Gram-negative and show a characteristic beaded appearance when stained by the ordinary stains. Growth is obtained at 37° C. on serum-agar under anaerobic conditions. The colonies are small, white opaque discs, with projecting wavy filaments. Cultures yield a characteristic "cheese-like" odour.

Rabbits and mice are highly susceptible to inoculation. Subcutaneous injection in rabbits produces an initial focus of necrosis at the site of inoculation and the animal dies in one to two weeks.

For diagnostic purposes stained films made from the edges of the necrosed tissue are examined.

Direct cultivation is difficult owing to the large numbers of other organisms present in the lesions. Pure cultures can be obtained readily by inoculating rabbits or mice from the necrotic tissue and isolating the organism on serum-agar from the inoculated animal at autopsy.

This organism has occasionally been isolated from suppurative lesions in the human subject.

CHAPTER XIX

THE PATHOGENIC AND COMMENSAL SPIROCHAETES

TREPONEMA PALLIDUM (SPIROCHAETA PALLIDA)

THE causative organism of syphilis.

Morphology.—An exceedingly delicate, spiral filament $6-14\mu$ (av. 10μ) by 0.25μ , with six to twelve coils which are comparatively small, sharp and regular. The length of the coils is about 1μ and the depth $1-1.5\mu$. The ends are pointed, and tapering. The organism is feebly refractile, and can be demonstrated in the unstained condition only by dark-ground illumination (*vide* p. 58). Multiplies by transverse fission.

The spirochaete shows rotatory corkscrew-like motility, and also movements of flexion. The coils remain relatively rigid, but there may be some expansion and contraction. Its progression is relatively slow as compared with many of the motile bacteria.

Staining.—*Treponema pallidum* cannot be demonstrated by the ordinary staining methods. It can be stained by Giemsa's solution applied in a 1 in 10 dilution over a prolonged period (twenty-four hours) or in a 1 in 2 dilution for an hour, and appears faint pink in colour. For the demonstration of this organism in films, Fontana's silver impregnation method (*vide* p. 171) is one of the best available in routine work. In tissues, the spirochaetes can be demonstrated by Levaditi's silver impregnation method (p. 174).

Culture.—Has been grown anaerobically by Noguchi and others, but artificial cultivation is only rarely successful. Noguchi's technique of isolating pure cultures was as follows. Deep tubes of 2 parts agar

and 1 part ascitic fluid were prepared, with a piece of sterile tissue added (*e.g.* rabbit kidney). The medium was covered with a layer of sterile vaseline. The tubes were inoculated from exudate with a capillary pipette (stab inoculation). Both the spirochaetes and the bacteria present in the material grew along the line of inoculation, but later, the spirochaetes spread out from the stab and formed a haze in the medium. The tube was cut, and transplants were made in Smith-Noguchi medium (*vide* p. 118), without carrying over any of the other bacterial growth. In this way pure cultures were obtained.

Occurrence.—In the primary stage, spirochaetes are present in large numbers in the chancre and in the exudate from it, but as the sore tends to heal they become less numerous, and may not be demonstrable in the exudate. They are present also in the buboes.

In the secondary stage, spirochaetes have invaded the blood stream and become widely distributed in the body. They are present in the roseolar skin lesions, mucous patches and condylomata, and have been demonstrated even in the blood.

In the tertiary stage, they are less easily demonstrated in lesions, but can be observed by suitable staining methods in the periphery of gummata, in arterial lesions, etc.

In general paralysis of the insane, *Treponema pallidum* has been demonstrated in the cerebral cortex.

In congenital syphilis, spirochaetes are found in certain internal organs—*e.g.* liver—often in very large numbers. They are present also in the skin lesions, the blood, and the mucosa of the intestine and bladder.

Spirochaetes have also been demonstrated in the placenta.

Animal Inoculation.—Monkeys have been infected experimentally by inoculation of a scarified area on the eyebrows and genitals. The anthropoid apes are the most susceptible, and lesions typical of primary and secondary syphilis may result in these animals. Rabbits can also be infected in some cases by inoculation in certain sites: inoculation into the

anterior chamber of the eye produces keratitis and iritis; intratesticular injection leads to a syphilitic orchitis; and inoculation of the skin of the scrotum may set up a chancre-like sore. Sometimes metastatic lesions may succeed the primary infection.

SEROLOGICAL REACTIONS

Wassermann Reaction.—Reference has been made to this manifestation of syphilis on pp. 204–218, and the technique has been described.

The reaction appears in about two to four weeks after the onset of the primary lesion, but its development may sometimes be delayed. In secondary syphilis the reaction is usually well marked and fairly constantly present (95 per cent. of untreated cases). It is less frequently positive in the latent stage (25–50 per cent. of cases) and in tertiary cases (75 per cent.). In general paralysis and in many cases of locomotor ataxia, the reaction is positive both when tested with blood and with cerebro-spinal fluid. In cerebro-spinal syphilis the spinal fluid may react positively even when the blood yields a negative reaction. Active cases of congenital syphilis usually exhibit a strongly positive reaction.

As the result of antisyphilitic treatment the Wassermann reaction may become negative, but often temporarily, and the reaction reappears when treatment is stopped.

Positive reactions have been recorded in leprosy and malaria, but in assessing the significance of these results, it must be remembered that concomitant syphilis may be responsible for Wassermann reactions noted in other diseases. The available evidence indicates that a positive reaction may develop in leprosy apart from associated syphilis. A positive Wassermann reaction occurs in Yaws, and has been recorded in cases of Relapsing Fever and Trypanosomiasis.

The Flocculation Reaction is dealt with on p. 219. It follows very closely the Wassermann reaction in its occurrence, and corresponds quantitatively with the complement-fixation reaction.

The "Sigma" and the "Kahn" reactions are flocculation tests in which the technique of the original reaction of Sachs and Georgi has been modified.

The Meinicke reaction is somewhat similar, and is described on p. 222.

DIAGNOSIS

In the primary stage, when there is an ulcerated sore, *Tr. pallidum* can usually be demonstrated in the serous exudate from the ulcer. The dark-ground illumination method is the ideal technique for the purpose, and provides a convenient means of rapid diagnosis. Failing this, Fontana's staining method can be used.

Tr. pallidum is recognised by its special morphological features, and must be carefully differentiated from other spirochaetes found in ulcerating sores—e.g. *Tr. gracile*, etc. (*vide infra*).

Directions for obtaining a specimen of exudate from a syphilitic sore for microscopic examination:

The serous exudate should be obtained from the tissue, and should not include surface organisms, as other spirochaetes which may be confused with the *Tr. pallidum* are frequently present. The presence of excessive numbers of red blood corpuscles in the specimen is also to be avoided, as they tend to obscure the spirochaetes. If a local antiseptic has been used it may not be possible to find spirochaetes until a wet dressing of gauze soaked in sterile saline solution has been applied to the sore for twenty-four to forty-eight hours. It is to be noted also that antisymphilitic treatment, initiated before the examination, diminishes the likelihood of successful microscopic diagnosis.

The sore is cleansed with a swab soaked in warm saline solution, and the margin is then scraped lightly with some blunt instrument to abrade the superficial epithelium. On squeezing the base of

the chancre, serum exudes, and if blood-stained, should be removed with dry gauze until clear exudate can be obtained. Some of this is then collected in one or two capillary tubes. Both ends of the tubes are sealed in the flame, and the specimens are submitted for examination. Another method of obtaining exudate is to apply spirit to the sore for a minute, wash it off with saline solution and allow the surface to dry; this leads to an exudation of serous fluid, which is collected and examined.

When the primary sore is in process of healing, microscopic examination of the exudate may yield negative results. At this stage spirochaetes may be found in the fluid aspirated from the buboes by means of a syringe.

In the secondary stage spirochaetes may also be demonstrated in the serum from the skin eruption, and in the exudate from mucous patches, etc. Serum can be obtained from the skin eruption by scarifying and "cupping" with a test-tube.

After about two weeks from the onset of the primary sore, the Wassermann reaction can be employed for diagnosis. The reaction becomes progressively more pronounced with the advance of the disease, and is usually markedly positive in the secondary stage.

Owing to the fact that the reaction may be slow in developing, if at first a negative result is elicited in the primary stage, it is essential to repeat the test before excluding syphilis. A negative reaction in a suspected case of secondary syphilis is highly significant in excluding syphilitic infection, but in supposed latent or tertiary cases a negative result does not exclude the disease.

In cerebro-spinal syphilis both the blood and spinal fluid should be tested.

In dealing with cases of congenital syphilis in young infants, the mother's blood should be tested if there is any difficulty in obtaining a specimen of blood from the child.

It may be said that the Wassermann reaction, carried out by a reliable technique, is clinically specific for syphilis among the diseases of a temperate climate.

The Flocculation reaction may for routine purposes be substituted for the Wassermann test, and is simpler to carry out.

TREPONEMA PERTENUE

The causative organism of Framboesia or Yaws, a tropical disease pathologically and clinically resembling syphilis, though differing in its highly contagious character.

Morphology.—Practically identical with the *Tr. pallidum*. When first described it was regarded as more delicate than *Tr. pallidum*—hence the designation.

Its occurrence in lesions corresponds to that of the *Tr. pallidum*, and the diagnosis of the infection is carried out as in syphilis, by demonstrating the spirochaete in the papules or ulcers. The Wassermann reaction is positive.

The infection is experimentally transmissible to monkeys and rabbits as in the case of syphilis. Certain differences have been noted in the experimental lesions as compared with those produced by *Tr. pallidum*.

Treponema gracile.—This organism may occur in the secretions of the genitals, and morphologically resembles *Tr. pallidum*. Its differentiation from the latter is therefore of practical importance in syphilis diagnosis. It is not usually found if care has been taken to obtain serum from below the surface of the chancre (*vide supra*). It is thicker than *Tr. pallidum* and its spirals are narrower; by the dark-ground illumination method it appears “glistening,” whereas *Tr. pallidum* is “dead white”; it stains more readily than *Tr. pallidum* by Giemsa’s method.

Treponema microdentium.—This organism flourishes in carious teeth, and may be found in the secretion between the teeth. It closely resembles *Tr. pallidum*

in morphology, but is shorter ($3-10\mu$), and the coils are shallower. It is more easily stained by the ordinary methods than *Tr. pallidum*.

Treponema mucosum.—Similar to *Tr. microdentium* in morphology, but is stated to have the property of producing mucin.

Treponema macrodentium.—Occurs in the mouth like *Tr. microdentium*. It also resembles *Tr. pallidum*, but is larger and thicker, with larger and less regular coils. It is more easily stained than *Tr. pallidum* and is coloured blue by Giemsa's method.

SPIROCHAETES OF THE "REFRINGENS" TYPE

Borrelia (or *Spironema*) *refringens*

These are large, motile, refractile spirochaetes (about $10-30\mu :: 0.5-0.75\mu$) with irregular wide and open coils which are relatively few in number. They are easily stained by the ordinary methods and are Gram-negative. They occur as commensals on various mucous membranes e.g. mouth and in gangrenous and ulcerative conditions on the surface of the body, the mouth and throat, and the genitals. A spirochaete of this form occurring in the mouth has frequently been designated by the specific name *buccalis*. This type of organism may also be found in the surface exudate of a syphilitic sore, and has to be differentiated morphologically from the *Tr. pallidum*.

"*Borr.* (or *Sp.*) *vincenti*" occurs in a pseudo-membranous condition of the throat—Vincent's angina. It resembles *Borr. refringens*, but is sometimes smaller ($5-10\mu$) and more delicate. It is generally associated with a large fusiform bacillus—*B. fusiformis* (*vide* p. 409). Films from the throat secretion, stained by dilute carbol fuchsin, show large numbers of spirochaetes.

A similar spirochaetal infection associated with *B. fusiformis* occurs in Balanitis gangrenosa, ulcerative gingivitis and a chronic ulceration of the skin of tropical countries (ulcus tropicum).

Similar spirochaetes have also been observed in putrid bronchitis and empyema, gangrene of the lung and pulmonary abscess.

THE SPIROCHAETES OF RELAPSING FEVER

BORRELIA (or SPIRONEMA) OBERMEIERI (or RECURRENTIS)

The causative organism of European relapsing fever.

Morphology and Staining.—This organism is a spiral filament, varying in length, as a rule, from $10-20\mu$, and about 0.3μ broad, with about five to seven fairly regular coils $2-3\mu$ long by 1μ in amplitude. Active motility of a rotatory or oscillating type is noted in fresh preparations. Multiplication is by transverse fission.

This spirochaete stains readily with a Romanowsky stain (*e.g.* Leishman's), and may exhibit uniform staining or beading. It can be stained also with carbol fuchsin, and is Gram-negative. In fresh preparations of blood it can be seen with the ordinary microscope, but dark-ground illumination is more suitable for its demonstration in the living state. Silver impregnation methods may also be used for demonstrating the spirochaete in films or tissues (*vide p. 171*).

Culture.—Artificial cultures were first obtained anaerobically in Smith-Noguchi medium, citrated blood containing spirochaetes from an infected animal—*e.g.* a white rat—being used as the inoculum.

More recently cultures have been obtained in other media : (1) horse serum diluted with 2 parts of saline solution containing 1 per cent. peptone bouillon and (for subcultures) a drop of rabbit blood, the medium being covered with a paraffin seal ; (2) 20 per cent. rabbit serum with 80 per cent. Hartley's broth in tubes to each of which 1 gram of coagulated egg albumin is added ; a vaseline seal is superimposed and the cultures are incubated at 30°C .

Occurrence.—This organism is present in the peripheral blood during the pyrexial stage of the illness,

and can be detected in blood films. When defervescence occurs it disappears from the blood, but may still be present in considerable numbers in the spleen, where it is phagocytosed by large mononuclear cells.

It is transmitted from person to person by the body louse, the *Pediculus corporis*. After this insect has sucked blood from an infected individual the organisms are demonstrable in the stomach for a day, and then disappear. They reappear after about six days in the body cavity and become widespread throughout the body of the insect. Infection results either through the contamination of the bite wound with the infective excreta of the louse, or by the crushing of the infective lice with the fingers in the act of scratching, and by the simultaneous inoculation of the abrasions.

Animal Inoculation.—Monkeys, white mice and white rats can be infected experimentally by subcutaneous injection of blood from a case of relapsing fever.

BORRELIA (or SPIRONEMA) DUTTONI

The organism of West African relapsing fever (African Tick Fever).

This organism is morphologically and biologically similar to *Borr. obermeieri*, but probably represents a separate variety or species. Its distribution in the disease is also similar, but it is transmitted by a tick (*Ornithodoros moubata*). Infection probably results from the contamination of the bite-wound by the infective excreta of the insect. It has been suggested that in the tick the organism goes through some stage in a life-cycle. "Chromatin" granules have also been observed in the spirochaete; these are extruded, and have been regarded as a phase in the life-history of the organism. These granules have been noted in the Malpighian tubules of infective ticks. Infectivity may be transmitted to the second generation from the female insect.

Borr. duttoni is pathogenic to monkeys and various

laboratory animals (guinea-pig, rat, mouse, etc.). It possesses a greater virulence for monkeys and other animals than *Borr. obermeieri*.

The spirochaete of North American relapsing fever resembles *Borr. obermeieri*, but apparently constitutes a separate species as judged by immunity reactions. It is designated *Borrelia* (or *Spironema*) *novyi*. It is louse-borne.

The organism of Indian relapsing fever also corresponds in its biology and pathogenesis to *Borr. obermeieri*. It has been regarded as a distinct species and named *Borrelia* (or *Spironema*) *carteri*, but it is doubtful if the Indian strains can be differentiated from the European. This infection is also louse-borne.

Louse-borne spirochaetal relapsing fever similar to the Indian form occurs in various parts of Asia, but in Central Asia ticks of the genus *Ornithodoros* are responsible for transmission.

The common form of relapsing fever in North Africa is louse-borne. In Tropical Africa the prevalent type is tick-borne (African tick fever, *vide supra*), though louse-borne infections occur in West Africa.

In Central and South America both louse-borne and tick-borne forms of the disease have been observed.

DIAGNOSIS OF RELAPSING FEVER

During the pyrexial phases, the spirochaetes can frequently be demonstrated in the blood, but not during the apyrexial intervals.

Thin or thick blood films are made as in malaria diagnosis, and stained by Leishman's method (*vide pp.* 168, 443).

Some workers prefer to stain the films with dilute carbol fuchsin.

If a drop of blood is mounted on a slide under a cover-glass and examined with the oil-immersion lens, the spirochaetes may be detected in the unstained condition and show active movement. A better

method of demonstrating them, however, is by dark-ground illumination.

If spirochaetes are not detectable, inoculation of white mice with blood drawn from a vein may reveal the infection, the organisms appearing in considerable numbers in the animals' blood.

BORRELIA (or SPIRONEMA) THEILERI

This spirochaete is responsible for a blood infection occurring in cattle, sheep and horses in Africa. The disease is of a comparatively mild type. The organism appears as a spiral filament $10-30\mu$ by $0.25-0.3\mu$ and is actively motile when seen in fresh preparations of blood. It is transmitted by a tick (*Margaropus decoloratus*).

BORRELIA GALLINARUM (or SPIRONEMA ANSERINUM)

This organism produces a general blood infection in geese and fowls. It can be seen in the blood of the infected bird both in unstained preparations and in films stained by a Romanowsky stain or dilute carbol fuchsin. It is a motile spiral organism $10-20\mu$ in length by 0.3μ in breadth and exhibits several coils. Artificial cultures have been obtained in Smith-Noguchi medium. The disease is transmitted by ticks—e.g. *Argas persicus*—and a granular phase has been described analogous to that observed in the case of *Sp. duttoni* (*vide supra*). By experimental inoculation various species of birds may be infected, but mammals are not susceptible.

LEPTOSPIRA ICTEROHAEMORRHAGIAE

The causative organism of Infectious Jaundice (Weil's Disease).

Morphology and Staining.—A leptospira (*vide p. 12*) about $7-14\mu$ long by 0.25μ broad. The coils are very numerous and so fine that they are difficult to demonstrate in stained preparations, though quite obvious by dark-ground illumination. In addition to these "elementary" spirals secondary curves may be seen, especially in stained films. Hooked ends are a characteristic morphological feature. In culture the organism tends to be longer than in the tissues. Active movement is observed in fresh preparations

examined with the dark-ground microscope. The movement is both rotatory and undulating.

The organisms can be demonstrated by Giemsa's stain (as in the case of *Tr. pallidum*) and by the silver impregnation methods of Levaditi and Fontana.

Culture.—*L. icterohaemorrhagiae* can be cultivated readily in Noguchi's leptospira media (*vide* p. 120), and grows just below the surface. The optimum temperature is from 25°–30° C.

Occurrence.—The organisms are present in the blood during the first six days of the illness, and though scanty, can in some cases be demonstrated microscopically in blood films. Later they disappear from the blood. They are present in the liver often in considerable numbers, and, particularly in the later stages of the disease, in the kidneys, when they can be detected in the urinary sediment.

Leptospira icterohaemorrhagiae occurs in wild rats and field-mice, which act as carriers of the infection. In these animals the spirochaetes are present in the kidneys, and are excreted in the urine. In this way soil, food, etc., are contaminated. Infection of the human subject may occur by the alimentary tract, but it has been shown that the organisms can pass through the skin, possibly more readily when it has become sodden by continuous wetting. In Japan, epidemics of Infectious Jaundice have been specially noted among workers in wet mines. Similarly during the War outbreaks occurred among troops in wet trenches. The disease has also affected miners in this country working in wet mines. The contamination of soil by the urine of rats harbouring the leptospira is undoubtedly an important factor in the transmission of the disease, and invasion occurs through skin abrasions or the intact skin.

Saprophytic leptospiras similar in morphology to *L. icterohaemorrhagiae* have been observed in water (e.g. *L. biflexa*), and the question has arisen whether these organisms are potentially pathogenic. Infectious jaundice has been produced in experimental animals

by inoculation with apparently saprophytic leptospiras, but generally such organisms have proved non-pathogenic.

L. icterohaemorrhagiae also infects dogs, producing the condition known as "Yellows," which is pathologically similar to human infectious jaundice.

The infection has also been observed in the fox.

DIAGNOSIS

During the first six days of the disease 5 c.c. of blood are withdrawn by vein puncture and injected intraperitoneally into a guinea-pig. The animal dies in eight to twelve days, showing a marked jaundice with haemorrhages in the lungs, under the serous membranes and in the muscles. The leptospiras are present in large numbers in the liver and kidneys, and can also be found in various other organs and in the blood.

The methods of demonstration in the stained and unstained conditions have been referred to above.

If a case is met with only at a later stage—e.g. after ten days from the onset—the urine is centrifuged and the sediment examined by the dark-ground microscope. Guinea-pigs should also be inoculated intraperitoneally with the centrifuged deposit.

The organism is present in the urine by the twentieth day in nearly every case; it may persist till the fortieth.

In the urine the organism often presents an atypical appearance and cannot be identified readily as a leptospira; this has been attributed to the bile or acid in the urine. Inoculation of animals with urinary sediment may not produce the characteristic disease; jaundice may be absent though lung haemorrhages are a more constant feature. In such animals the leptospiras may be relatively scanty in the tissues.

It may be noted here that the elementary coils of the dying organism may become unwound or opened out, so that it presents an appearance more like a treponema.

Rieckenberg Reaction (Adhesion phenomenon, thrombocytobarin reaction).—This reaction has been applied in the serological differentiation of races of spirochaetes and by this means *L. icterohaemorrhagiae* can be distinguished from

L. biflexa and *L. hebdomadis* (*vide infra*). When spirochaetes are acted on by a specific antiserum they undergo such change that particles present (e.g. blood platelets, bacteria, etc.) tend to adhere to them, the adhesion being easily observable by means of the dark-ground microscope. The test is carried out by mixing the antiserum with a fluid culture of the spirochaete and then adding a suspension of *B. coli*; the mixture is incubated at 30° C. for twenty minutes and a microscopic preparation is then examined by dark-ground illumination. The adhesion of the bacteria to the spirochaetes constitutes a positive reaction.

LEPTOSPIRA HEBDOMADIS

An organism described as the cause of "seven-day" fever of the East. It is morphologically identical with *L. icterohaemorrhagiae*. In some cases it has been demonstrated in the peripheral blood, and at a later stage of the illness appears in the urine. It is pathogenic for young guinea-pigs, producing a febrile illness rarely associated with jaundice, and with less tendency to haemorrhages than in other leptospiral infections. These animals may also recover from the infection. This organism has been shown to differ serologically from the other pathogenic leptospires. It is harboured by a field mouse (*Microtus montebelloi*) and excreted in the urine as in the case of rats infected by *L. icterohaemorrhagiae*. The mode of infection is probably similar to that in infectious jaundice.

Leptospira autumnalis.—This organism has been found associated with a disease in Japan called Akiyami, or harvest sickness, and clinically resembling a mild infectious jaundice. Cases may be confused with "seven-day fever" but *L. autumnalis* can be distinguished from *L. hebdomadis* by its high infectivity to guinea-pigs, in which it produces typical haemorrhagic jaundice. It can be differentiated from *L. icterohaemorrhagiae* by serological reactions. The field mouse (*Microtus montebelloi*) is probably the reservoir of this infection.

LEPTOSPIRAS DESCRIBED IN PHLEBOTOMUS FEVER (SAND-FLY FEVER), DENGUE, AND SIMILAR DISEASES

It has been claimed that phlebotomus fever is due to a leptospiral organism which has been isolated from the blood of cases by cultivation in Noguchi's leptospira medium (Whittingham). The organism proved non-pathogenic to guinea-pigs. It has long been recognised that a sand-fly (*Phlebotomus pappatasi*) is the vector of the specific virus, and the experimental work on the disease points to its causation by a filterable virus.

A leptospira has also been described in Dengue. The organism was demonstrated in the blood at the commencement of the illness, and was found to be pathogenic to rabbits, producing pyrexia and blood infection. It has been stated that the injection of rabbits with crushed infective sand-flies produces a similar disease, and that the inoculated animals show the spirochaetes in their blood. Dengue is now generally regarded as a filterable virus disease.

It seems probable that in the Mediterranean area and Near East leptospiral infections occur which simulate Phlebotomus fever and Dengue. These may be *L. icterohaemorrhagiae* infections of a mild type with absence of the typical jaundice, or may be due to different races of *Leptospira* of lower virulence for man and laboratory animals than *L. icterohaemorrhagiae*.

A leptospira (*L. pyrogenes*) has been reported in a febrile disease occurring in Sumatra associated with some degree of jaundice and albuminuria. This organism is pathogenic to guinea-pigs, but its effects are irregular. It has been isolated by cultivation from the blood. *L. pyrogenes* has been found to be serologically distinct from other races of leptospira.

SPIROCHAETA MORSUS MURIS (SPIRILLUM MINUS)

The causative organism of rat-bite fever. Though originally described as a spirochaete, this organism conforms in its biological characters to those of a spirillum, and the alternative name *Spirillum minus* has been used.

It is a short spiral organism about 2-5 μ in length and relatively broad with regular short coils numbering one for each micron of the length of the organism. Longer forms up to 10 μ may also be observed. This organism is very actively motile, showing darting movements like those of a vibrio. Movement is due to terminal flagella, which are variable in number—from one to seven at each pole. In moving, the organism itself remains rigid and shows no undulation. It can be demonstrated easily by dark-ground illumination in fresh preparations in which its active movement is seen and its flagella are also observed. It is most readily stained by a Romanowsky stain (e.g. Leishman's), but can also be stained by the ordinary aniline dyes.

Successful cultivation has been claimed by certain workers using Smith-Noguchi and Shimamine medium. The latter is prepared by adding 0.5 gm. sodium nucleate to 160 c.c. of sterile horse serum; carbon dioxide is passed through the mixture for a few minutes until the medium becomes transparent; it is then heated on three successive days at 60° C., and on the fourth day at 65° C. for ten minutes, when it becomes semi-coagulated.

In rat-bite fever the spirillum may be demonstrated in the local lesion, the regional lymph glands, and even in the blood, either by direct microscopic methods (*vide supra*) or by animal inoculation. Guinea-pigs, white rats and mice are susceptible to infection: the spirilla appear in the peripheral blood and can be detected easily by dark-ground illumination. Guinea-pigs develop a progressive disease and die of the infection; in mice the organisms gradually disappear from the blood without producing, as a rule, a lethal effect. If the spirillum cannot be detected microscopically in the local lesion, or if the original bite wound has healed, an enlarged lymphatic gland may be punctured by means of a hypodermic syringe; "gland juice" is aspirated and investigated by direct methods or animal inoculation.

Spirillum minus occurs naturally in wild rats and certain other wild rodents, producing a blood infection. A disease similar to rat-bite fever has also been reported following a ferret-bite.

CHAPTER XX

RICKETTSIA GROUP ("RICKETTSIA BODIES"); BARTONELLA (OROYA FEVER)

THE generic name *Rickettsia* is applied to a group of organisms whose biological relationships are still unknown, though they may be classified provisionally with the bacteria. These organisms flourish in the alimentary tract of certain blood-sucking insects (*e.g.* lice, bugs and ticks) and several species have now been described. Some have no relationship to human or animal disease, but certain can be definitely incriminated as pathogenic to the mammalian host on which the insect-carrier is parasitic. The insect acts as the vector of the infection. Thus *Rickettsia prowazeki* constitutes the specific virus of typhus fever; it is found in lice which have fed on typhus cases, and this insect is the vector of the disease.

While investigating the cause of typhus fever, Ricketts and Wilder found in the alimentary tract of infected lice small diplococcal or rod-shaped structures, about 1μ or less in their long diameter, which stain reddish or purple by Giemsa's method. These small bodies, now termed *Rickettsiae* (or "*Rickettsia* bodies"), have been found in infected insects transmitting other diseases such as Trench Fever and Rocky Mountain Spotted Fever, and are regarded as the aetiological incitants of these diseases. Although *Rickettsia* bodies are found constantly and in large numbers in infective insects, and infectivity coincides with their presence, similar bodies have been found in non-infective insects such as lice, but in relatively small numbers and very infrequently. (It was for this reason that Ricketts and Wilder hesitated to regard the *Rickettsia* as the cause of typhus fever.) It seems probable that these *Rickettsiae* represent non-pathogenic species. In addition similar bodies

have been found in the sheep "ked" (*Melophagus ovinus*) and have been termed *Rickettsia melophagi*, while the bed-bug (*Cimex lectularius*) almost constantly contains such bodies in the intestinal tract. There is no doubt that *Rickettsia* bodies are definite living structures capable of multiplication, and that their presence in the insect may be associated with infective powers. They have not yet been cultivated on food media, except *Rickettsia melophagi* which appears to multiply on blood-agar.

TYPHUS FEVER

This disease is transmitted by the louse *Pediculus humanus* (*corporis* and *capitis* varieties), and the specific virus is now generally accepted to be *R. prowazeki* (*vide supra*). The disease can be reproduced in monkeys by inoculation with blood from human cases. The virus can also be communicated to guinea-pigs by the intraperitoneal injection of 3-5 c.c. of patient's blood taken during the height of the illness. After an incubation period of about nine days, the animal's temperature rises to 105° F., and remains above the normal for four to eight days. The guinea-pig does not succumb to experimental typhus infection, and on recovery does not react to a second injection of virus. If the animal is killed on the first or second day of the fever, the heart blood is infective for other guinea-pigs, but does not contain any recognisable bacteria. At autopsy, the spleen is enlarged and the peritoneum covering it is dull in appearance. The skin, on microscopic examination, shows the presence of an exanthematous reaction.

The virus is demonstrable (by experimental inoculation) in the blood, spleen, brain and other organs. It is easily destroyed by heat and antiseptics, but is resistant to drying. It is not filterable.

Rickettsia prowazeki.—Small diplococcal or rod-shaped bodies, usually 0.3-1 μ , but are pleomorphic and may attain a length of 4-20 μ . They are non-motile and stain reddish-purple with Giemsa's stain. They stain very feebly with ordinary stains and are Gram-negative. Up to the present they have not been cultivated on artificial media, but multiply in endo-

thelial cells of a tissue culture. These bodies are found in the alimentary tract of lice infected with the blood of typhus fever patients and are regarded as the cause of the disease. They may be found in large numbers in smear preparations (stained by Giemsa's method) from the gut contents of the lice seven to ten days after infection. They invade the lining epithelial cells, which become packed with them. They are excreted in the insect's faeces, and infect the human subject through the bite-wounds or through scratches and other abrasions.

The experimental disease referred to above has been produced by inoculation with the gut contents of infective lice. Ricketts and Wilder claimed that they were able to demonstrate *Rickettsia* in the blood of cases, but their findings have not been confirmed. *Rickettsiae* are demonstrable microscopically however in the vascular endothelium of the skin, brain, etc., in the human disease and in the experimental infection. They have been observed in the endothelial cells of the tunica vaginalis of male guinea-pigs after intraperitoneal inoculation of the American typhus virus.

The serum of typhus patients contains agglutinins for a type of *B. proteus* designated "X 19," originally isolated from the urine of cases. This serological feature of the disease is a fairly constant one, and the agglutination reaction with this organism (Weil-Felix reaction) has therefore been utilised for diagnosis. The reaction is not present in other conditions. The immunological significance of the phenomenon has not yet been elucidated, but is not to be interpreted as indicating an aetiological relationship of *B. proteus* "X 19" to the disease.

DIAGNOSIS

The Weil-Felix reaction is performed in exactly the same manner as the Widal test (p. 196), except that a suspension of *B. proteus* ("X 19") is used instead of the enteric organisms. The minimum titre acceptable for diagnostic purposes is 1 in 100, but agglutination frequently occurs in much higher dilutions—e.g. 1 in 1700. The reaction appears early in the disease (during the first week).

Inoculation of the guinea-pig may also be carried out (*vide supra*).

TYPHUS-LIKE INFECTIONS AND OTHER INFECTIONS DUE TO RICKETTSIAE

A typhus-like condition of America, Rocky Mountain Fever, has been shown to be due to a species of *Rickettsia*, designated *Dermacentroxenus rickettsi*. This organism is demonstrable in a tick *Dermacentor venustus* which transmits the disease. It is similar in appearance to *R. prowazeki*. *Rickettsia* bodies have been observed also in mononuclear cells in the human disease.

In India and the Far East typhus-like conditions occur which are frequently of a milder type than European typhus—e.g. Japanese Flood Fever (Tsutsugamushi) transmitted by mites, and regarded as due to a *Rickettsia* designated *R. orientalis*. To this category also belongs the so-called “rural” or “scrub” typhus of Malaya. A peculiarity of this disease is the fact that while the Weil-Felix reaction with *B. proteus* “X 19,” is absent, a variant of this organism (the Kingsbury strain) is agglutinated by the serum.

Trench Fever.—This condition was prevalent among troops during the War and like typhus was louse-borne. A *Rickettsia* (*R. quintana*) was found in lice fed on patients and has been regarded as the cause of the disease. Unlike *R. prowazeki* it does not invade the alimentary epithelium of the insect and is less pleomorphic.

Heart-Water of cattle in South Africa is a tick-borne disease and a *Rickettsia* (*R. ruminantium*) has been demonstrated in the vascular endothelium of infected animals and in the insect vector.

BARTONELLA BACILLIFORMIS (OROYA FEVER)

Oroya fever is a disease occurring in Peru, with a high degree of mortality, characterised by intermittent fever and anaemia.

The infective agent—*Bartonella bacilliformis*—invades red blood corpuscles and is found as minute rod-shaped forms 0·3–2·5 μ in length, while coccal forms also occur. It is actively motile. By Giemsa's method it stains reddish-violet in colour; the extremities appear thickened and darker in colour, and the outlines hazy. The organism is Gram-negative. Cultures are obtained in semi-solid leptospira medium and on solid or semi-solid blood media, but not in ordinary broth or agar. The optimum temperature is 25°–28° C. and the organism can survive in blood or in cultures for many weeks.

The disease can be reproduced by the intravenous inoculation of monkeys, except that anaemia does not occur.

Subcutaneous inoculation into the eyebrows of a monkey also produces nodules in which numerous organisms are present.

In *Verruga peruviana*, a disease characterised by a nodular eruption, an organism closely resembling *Bartonella bacilliformis* has been isolated. Noguchi's work has proved that these organisms are serologically identical, and that Oroya fever and *Verruga peruviana* have the same aetiological origin.

It seems possible that the organism described as *Anaplasma* (*vide* p. 446) in an infection of cattle and found also in red corpuscles is related biologically to *Bartonella*.

Bartonella muris.—It has been observed that after splenectomy in rats, organisms of the same type as *Bartonella bacilliformis* frequently appear in the red cells and this infection is associated with marked anaemia and often produces a fatal result. The organism has been named *Bartonella muris*. Presumably latent infection is prevalent in rats associated with a certain degree of natural immunity which is broken down by the removal of the spleen. A similar organism has also been noted in mice following splenectomy.

CHAPTER XXI

THE FUNGI

INFECTIONS produced by the fungi (*vide* p. 4) are usually designated "mycoses."¹

The more important of these are due to organisms of the group of so-called **FUNGI IMPERFECTI**—*i.e.* fungi which do not possess characteristic fruiting organs and show no complete life-cycle. This group includes *Microsporon*, *Trichophyton*, *Achorion*, etc.

MICROSPORON

This organism is the common cause of scalp ring-worm in children. It rarely attacks the body. It is apparently unable to infect the scalp after puberty.

The mycelium develops in the horny layer of the scalp epidermis, and in the hair medulla, from which hyphae pass through the cortex, and produce a covering of small spherical spores (approximately 2μ in diameter) arranged in the form of a mosaic on the outside of the hair. Grown artificially, *Microsporon* develops large circular colonies with a raised centre and radiating folds. The surface of the colonies is velvety, due to the projecting aerial hyphae. Pigmentation is often marked, and the actual colonies become brown in colour though the surface layer remains white or buff-coloured.

¹ For detailed information on this subject reference may be made to "Fungi and Fungous Diseases," by A. Castellani (Adolph Gehrman Lectures, University of Illinois College of Medicine, 1926; reprinted from *Archives of Dermat. and Syph.*, October 1927–March 1928).

Reference may also be made to "Fungi pathogenic to Man," *A System of Bacteriology*, Medical Research Council, 1931, vol. viii., chap. 1.

Various species have been recognised—e.g. *M. audouini* (the common type in ringworm of the scalp among children), *M. canis* (dermatomycosis of dogs), *M. felineum* (of cats), *M. equinum* (of horses). Species of animal origin may infect the human subject—e.g. *M. felineum*.

TRICHOPHYTON

This organism may occur in ringworm of the scalp, nails and glabrous skin. It may also be found in beard ringworm.

The fungus mycelium, as it develops in the hair medulla or epidermis, produces characteristic chains or “rosaries” of oval or rectangular spores, which are larger ($3-5\mu$ or even 8μ) than those of *Microsporon*. In artificial culture the different species vary in detailed characters, but present the same general type of growth as *Microsporon*. A common species in this country occurring in scalp ringworm is *T. crateriforme*, which produces raised colonies with crater-like depressions. Some species are markedly pigmented—e.g. *T. rosaceum*, *T. violaceum*.

Different species associated with dermatomycoses in animals have been recognised: *T. equinum* (in horses), *T. caninum* (in dogs), *T. felineum* (in cats). *Trichophyton* infections in animals are not uncommon sources of human ringworm.

ACHORION SCHÖNLEINII

Associated with the condition called “favus,” which affects the scalp and other skin areas.

The fungus forms the characteristic concave yellow discs or “scutula” centred round hair follicles. The scutulum is composed of mycelium and spores, which are markedly irregular in size and arrangement. The hairs in the affected area are also invaded. The artificial growth on culture medium consists of a tough brownish layer with raised irregular folds, and a white velvety surface due to the aerial hyphae.

Achorion quinckeum is the species of mouse favus ; it is occasionally found in the human disease.

A favus-like condition occurs in fowls due to *Achorion gallinae*.

EPIDERMOPHYTON

This type of organism does not attack the appendages but grows in the superficial layers of the epidermis, and skin folds are specially susceptible. The infection is exemplified by *Tinea cruris* ("Dhobie itch"); a similar condition may occur between the toes (*Tinea interdigitalis*). A number of species of *Epidermophyton* have been recognised and differentiated mainly by the colour of growths on culture medium—e.g. *E. cruris* (yellow), *E. perneti* (pink), *E. rubrum* (deep red).

Microsporon furfur of pityriasis versicolor, *Endodermophyton concentricum* of *tinea imbricata* (in which the mycelial growth occurs between the rete Malpighi and stratum corneum of the epidermis), and *Endodermophyton castellanii* of *tinea intersecta* are further examples of "fungi imperfecti" responsible for human dermatomycoses.

Sporotrichon beurmanni.—Associated with subcutaneous ulcerating granulomata. The condition is designated "sporotrichosis" and occurs in man and horses. When grown artificially, the organism forms a typical mycelium ; the hyphae are relatively narrow (2μ) ; oval spores are found in clusters at the ends of hyphae. In the tissues it may be difficult to demonstrate the organism by microscopic examination ; the morphology is entirely different, and no definite mycelium is observed ; oval or spindle-shaped forms are found ; some of these are not unlike large bacilli ; budding may be noted as in the case of the blastomycetes.

This organism is pathogenic to mice and white rats, experimental inoculation of cultures producing an abscess at the site of injection.

Hemispora stellata.—The organism of hemisporosis, a granulomatous and suppurative condition, occurring in the human subject and involving the skin in the form of single or multiple lesions. The infection may also occur in bones. This fungus, which appears as a mycelium with star-like conidiophores, is easily cultivated from lesions as an irregular whitish layer on

the surface of the medium. It is pathogenic for the rabbit on experimental inoculation. It is not uncommon as a saprophyte and may be found in dust.

Allergic Reactions in Fungus Infections.—Deep-seated infections of the skin, especially those due to *Trichophyton*, may lead to widespread cutaneous hypersensitiveness to the products of the organism. Thus, in the course of the infection, generalised skin eruptions of an allergic nature may occur—e.g. the “trichophytides.” Evidence of such allergy may be elicited by injecting extracts of the organism—e.g. trichophytin.

Madurella, a hyphomycete found in mycetoma, is referred to on p. 408.

PHYCOMYCETES

Mycelium made up of non-septate hyphae ; asexual spores developed in a spherical “sporangium” or spore-case borne on the expanded end of an aerial hypha ; “zygospores” also formed as the result of conjugation of two hyphae at their tips. These fungi are of little or no importance as pathogens, though they have been noted in auricular, naso-pharyngeal and pulmonary mycoses, and in mycosis of the tongue. They are frequent contaminants of food, and of culture medium in the laboratory. The common mould *Mucor mucedo* exemplifies the group.

Rhinosporidium.—This organism has been classified with the Phycomycetes. It is responsible for a polypoid disease of the nose and tumour-like lesions of the conjunctiva, uvula and cheek occurring in India and South America—e.g. Argentine. In an early phase of its life-history it appears in the tissues as a small rounded capsulated body with a single nucleus. This cell enlarges and the cytoplasm and nucleus divide into a large number of “spores.” The parent structure, surrounded by a thick cellulose capsule, may reach 100 μ in diameter, and with the contained spores has been regarded as analogous to a sporangium. The spores ultimately escape through a pore in the capsule and spread in the lymphatic spaces.

ASCOMYCETES

Mycelium typically septate ; “ascospores” developed in a spore case (“ascus”) formed at the end

of a hypha. The moulds *Aspergillus* and *Penicillium* are examples of this group.

Aspergillus is a frequent contaminant of culture media, occurring as a felted yellow, green, or black mould-growth; rows of spores or "conidia" develop from finger-like processes or "sterigmata" borne on an expanded aerial hypha. Pulmonary aspergillosis may occur in birds—e.g. pigeons—and this infection has also been noted in man (among bird fanciers). *A. pictor* is responsible for a skin disease of Central America called Pinta, but other species of fungi are also associated with different varieties of this condition.

Penicillium glaucum is one of the commonest food moulds and contaminants of culture medium. Its biology is similar in many respects to that of *aspergillus*. Cultures assume a characteristic dull green colour.

SACCHAROMYCETES

(BLASTOMYCETES, YEASTS)

These are often classified with the Ascomycetes, and the yeasts are now regarded more as a phase of other types of fungi than as a separate group. Their morphology and gemmation, or budding, are highly characteristic features, and though in some species hyphae occur, it seems justifiable to consider them separately from the hyphomycetes or moulds.

Pathogenic types :

Cryptococcus : typical yeast-like forms; no hyphae occur; when grown in fermentable carbohydrates no gas is produced.

Cryptococcus gilchristi—of Blastomycetic dermatitis.

Cryptococcus linguae pilosae—of "Black-tongue."

Cryptococcus farciminosus of Epizootic Lymphangitis in horses.

The genus *Coccidioides* is characterised by the formation of mycelium, and ascospores have been noted.

Coccidioides immitis—of "Blastomycosis," an uncommon granulomatous or suppurative condition involving skin, subcutaneous tissue, lungs, etc., developing sometimes into a generalised pyaemic condition.

Monilia.—This type is also yeast-like in morphology and exhibits the characteristic gemmation, but hyphal filaments also occur. No ascospores have been noted. Various carbohydrates are fermented with both acid and gas production.

M. candida (*Oidium albicans*) is the species found in thrush. *M. psilosis* has been described as the causative organism of Sprue, but its aetiological relationship is doubtful.

M. tropicalis is the commonest type found in bronchial infections (broncho-moniliasis).

Monilia and *Cryptococcus* types have been shown to be the aetiological agents of dermatitis (in circumscribed form), suppurative paronychia and deformities of the nail-plate.

DIAGNOSIS OF THE DERMATOMYCOSES

The affected hairs, or the epithelial scales from affected areas, are treated on a microscope slide with a 10 per cent. solution of caustic potash, gently warmed, and examined (with a $\frac{1}{8}$ -in. lens) in the unstained condition under a cover-glass. The alkali clears the hairs and epidermis, and renders the fungi easily detectable under the microscope. Artefacts that may be mistaken by inexperienced observers for fungal elements, and therefore to be remembered in diagnostic work, are oil or fat droplets, air bubbles, spaces between epithelial cells, etc. Stained preparations are unnecessary for routine diagnostic work.

For artificial culture, Sabouraud's medium, standardised to pH 5.0 – 5.5 (*vide* p. 121), is a suitable medium, and it tends to restrain the growth of bacteria. A layer of the medium is solidified in the bottom of a conical flask. In making cultures from scales and hairs, two minutes' immersion in absolute alcohol before "planting" them on the medium assists in the isolation of the fungus by destroying the cocci and other bacteria that may be present on the skin. The mouth of the flask is covered with a rubber cap over the cotton-wool plug, and the cultures are incubated at 25°C. The growth of the fungus may be relatively slow, and the cultures may have to be incubated over a prolonged period to elicit characteristic appearances.

The tellurite trypsin agar as used for the isolation of the diphtheria bacillus (p. 113) is also suitable for the growth of ringworm fungi. The medium is inhibitory to skin organisms such as staphylococci and it enables pure growths to be

obtained from primary cultures. The ringworm hairs are treated with 50 per cent. alcohol for five minutes, and then placed on the surface of the medium in Petri dishes. To prevent evaporation a strip of $\frac{1}{2}$ in. adhesive plaster is placed round the circumference of the dish. Growths visible with a hand lens can be obtained after three to five days' incubation, while growths visible to the naked eye can be seen in a week to ten days (Allison).

CHAPTER XXII

MALARIA PLASMODIA; BABESIAE; TRYPANOSOMES; LEISHMANIAE

THE MALARIA PLASMODIA

MALARIA is a protozoal disease in which the causative organism—*Plasmodium*—invades the red cells of the blood. Three well-defined species of the malaria plasmodium are recognised :

P. vivax—Benign tertian malaria.

P. malariae—Quartan malaria.

P. falciparum—Malignant malaria.

These organisms belong to the order Haemosporidia of the Sporozoa. The plasmodium is transmitted by anopheline mosquitoes, and goes through the sexual phase of its life-cycle in the body of the insect.

Outline of the life-history of the Malaria Plasmodium.
—It is introduced into the body by the bite of the mosquito (*vide infra*) as a minute spindle-shaped cell or sporozoite, containing nuclear material in the form of a granule of chromatin. The sporozoite enters, or becomes attached to, a red cell, and assumes an amoeboid form (amoebula), which gradually develops (trophozoite). It displays amoeboid movement and protrudes pseudopodia. It grows at the expense of the red cell and accumulates altered blood pigment in the form of brownish granules. More than one trophozoite may attack a single corpuscle. The corpuscle may show considerable alteration in size as the trophozoite develops. The trophozoite tends to assume a characteristic ring-form, with the chromatin granule or “dot” at one side, so that the whole structure resembles a signet-ring. When full-grown it is more or less rounded, and may appear to occupy

the greater part of the corpuscle. These "mature trophozoites" usually contain a considerable amount of blood pigment.

When fully developed, the trophozoite undergoes schizogony. The pigment accumulates towards the centre, the chromatin breaks up into smaller particles, the protoplasm subdivides and forms a number of small round or oval merozoites (about 2μ in diameter) each containing a fragment of the original chromatin. The residual protoplasm and pigment remain in the centre of the group of merozoites. The number of merozoites resulting from schizogony varies with the different species (*vide infra*). Finally the individual merozoites are liberated as free structures in the blood. The merozoite attacks another red cell and thus the asexual cycle is repeated. The pigment is taken up by leucocytes.

The length of time the organism takes to complete the asexual cycle varies with the species:

<i>P. vivax</i>	.	.	.	.	Two days
<i>P. malariae</i>	.	.	.	.	Three days
<i>P. falciparum</i>	.	.	.	.	One or two days.

The fever develops at the stage of schizogony; thus in *P. vivax* infections the febrile paroxysm occurs every second day (tertian malaria); in *P. malariae* infections every third day (quartan malaria).¹

In malignant malaria, schizogony occurs as a rule only in the blood of internal organs, and not in the peripheral blood.

While some of the organisms develop into schizonts, others become gametocytes. In the case of *P. vivax* and *P. malariae*, these are rounded and about the same size as a mature trophozoite. The gametocytes of *P. falciparum* are sausage-shaped or crescentic, with the envelope of the corpuscles stretched across the

¹ The terms "tertian" and "quartan" are derived from the occurrence of the successive attacks on the third and fourth days respectively, counting the day of the previous attack as the first.

DIFFERENTIATION OF THE MALARIA PLASMODIA

	P. VIVAX (<i>Bonign tertian</i>)	P. MALARIAE (<i>Quartan</i>)	P. FALCIPARUM (<i>Malignant</i>)
Asexual life-cycle	48 hours	72 hours	24 to 48 hours
Fresh unstained preparation of blood	Amoeboidae { Not refractile, hyaline, not easily observed; usually one organism only in red cell	Refractile, "frosted-glass" appearance; more easily observed	Very small, about 1/4th of diameter of red cell; often more than one organism in cell
	Movement { Active	Not active	Active at first
Pigment	Fine, yellowish-brown	Coarse, brownish black	Scanty, fine
Ring forms (stained preparations)	Large, irregular, not well defined; usually single chromatin dot	Thick round rings, often in equatorial bands	Small, well defined, thin; often 2 chromatin dots; often flattened out on surface of red cell
Red cell (stained preparations)	Swollen, pale, showing deeply stained points, "Schuffner's dots"	Not altered	Shriveled, deeper colour, but may be swollen and pale
Schizont (stained preparations)	Large, mulberry-like; 12-24 oval merozoites	Small "daisy-head"-like; 6-10 round merozoites	Small; segmentation irregular; 8-32 very small merozoites; not seen in peripheral blood
Gametocytes	Rounded; macrogametocyte about one and a half times the size of red cell	Rounded; about the same size as red cell	Elongated, crescentic or sausage-shaped

poles of the crescent. Male and female gametocytes are distinguished, and designated respectively micro- and macro-gametocytes. As compared with the macro-gametocyte, the micro-gametocyte contains a nuclear structure which is richer in chromatin, and its pigment granules are finer. In the female gametocyte of *P. falciparum* the pigment is usually accumulated in the centre of the organism.

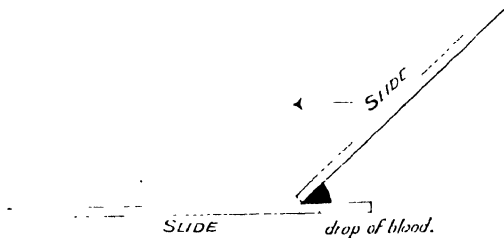
These gametocytes remain unchanged in the blood until it is withdrawn from the body—*e.g.* by the mosquito or when a drop is exposed to air and transferred to a warm stage for microscopic observation. In the stomach of the mosquito the following changes occur: the gametocytes of the crescent type become rounded; from the male cell 4 to 8 flagella-like structures are quickly protruded ("exflagellation"); these are long slender processes with somewhat enlarged free ends, each containing a chromatin granule derived from the parent nucleus, and are the microgametes; they are ultimately detached, and move with a kind of lashing motility; the macrogametocyte undergoes "maturation"—*i.e.* the protrusion and detachment of one or two polar bodies containing part of the original nuclear chromatin—and becomes the macrogamete; a micro- and macrogamete unite and form the zygote or oökinete, which slightly elongates and penetrates the stomach wall, embedding itself between the muscle fibres; it rounds itself later, forms an encysting membrane ("sporocyst") and increases in size until it projects into the body cavity; division into rounded "sporoblasts" occurs, and these divide again into the spindle-shaped sporozoites; the cyst ultimately ruptures and the sporozoites are set free in the body cavity and settle in the salivary gland, from which they are injected with the salivary secretion when the insect bites; this phase in the life-history is that of sporogony as contrasted with the asexual schizogony in the human subject. This stage takes seven to ten days (under favourable temperature conditions).

Plasmodium ovale (Stephens).—This is a fourth species or sub-species of malaria plasmodium, which has been reported in a few cases infected in East and West Africa. It resembles *P. malariae* but the erythrocytes are often markedly enlarged, oval in shape, show an irregular outline and exhibit the stippling (Schüffner's dots) as seen in the benign tertian form of malaria. The illness is tertian in periodicity. The infection has been transmitted experimentally by the bite of *Anopheles maculipennis*.

Certain other supposed sub-species of *Plasmodium* have also been described.

DIAGNOSIS

THIN BLOOD FILMS.—Two or three films are made on microscope slides or $\frac{3}{4}$ -in. square No. 1 cover-glasses



which have been carefully cleansed (*vide* p. 148) and polished with a smooth cloth.

Requisites.—Straight triangular needle, slides or cover-glasses, spirit lamp or Bunsen, gauze, methylated spirit.

The blood is obtained by puncturing the lobe of the ear, or the finger close to the base of the nail. The needle and the area to be punctured are cleansed with spirit.

Films on slides.—Touch the exuding drop of blood with the surface of a slide close to one end; place the narrow edge of a second slide kept at an angle of 45° on the drop of blood and allow the blood to spread out across the slide in this angle before drawing out the film; now spread the blood uniformly on the slide in the form of a thin film (see diagram).

Films on cover-glasses.—Touch the drop of blood with the surface of a cover-glass held by the edges between the thumb and first finger of one hand, and place a second cover-glass over the first so that the drop spreads out between them. Then at once take the second glass by the edges between the thumb and forefinger of the other hand, and slide the two cover-glasses apart without exerting pressure.

The films are allowed to dry, and are then fixed and stained with Leishman's stain (*vide* p. 168). They are mounted and then examined, first with a dry $\frac{1}{6}$ -in. lens, and later with the oil-immersion objective. In searching for crescents it is advisable to use the former.

THICK BLOOD FILMS.—As a routine measure, and especially when the organisms are likely to be scanty, thick films should be prepared and examined. A large drop of blood is deposited on a slide, and spread with the head of a pin in the form of a *thick* film about $\frac{3}{4}$ in. in diameter; it is thoroughly dried—*e.g.* in the incubator—and the haemoglobin is removed by treating with acid-alcohol (alcohol 50 c.c., hydrochloric acid 10 drops) and then washing in water; the film is stained by Leishman's method.

FRESH PREPARATIONS OF BLOOD FOR MICROSCOPIC EXAMINATION.—A drop of blood is deposited on a slide, covered with a cover-glass, and the edges of the glass are smeared with vaseline to prevent drying of the film. A warm-stage apparatus should be used during the microscopic examination.

Only stained films are examined as a rule in routine work. They should, if possible, be taken during the pyrexia. The organisms can be recognised by their various characteristic appearances, and it may be possible to determine the species or type present (*vide* Table, p. 441). It must be remembered, however, that the young ring-forms of the three types may be almost indistinguishable from one another and if only young rings are present in the film, it may

be difficult to determine the species. To inexperienced workers, artificial appearances may sometimes simulate malaria parasites, and a blood platelet overlying a red corpuscle may be mistaken for a young form of the plasmodium. In certain cases prolonged search may be required. At least two hundred microscope fields should be examined before the specimen is reported as negative. The absence of parasites during an apyrexial interval by no means excludes malaria. It is advisable, in searching for scanty malaria organisms, to examine particularly the edges of the film. They may be more numerous there than in the centre.

Certain *cytological features of the blood* may, in the absence of actual organisms, suggest the existence of a malarial infection—*e.g.* a relatively high percentage of large mononuclear cells (15–20 per cent.), or the presence of leucocytes containing altered blood pigment (*vide supra*).

THE PIROPLASMS (or BABESIAE)

These protozoal organisms (classified with the Sporozoa) produce disease in various domesticated animals, but are not known to infect the human subject.

They invade red blood corpuscles like the malaria plasmodia, and multiple infection of these cells is characteristic. The individual organisms are pyriform bodies about 2 to 4μ in length, containing a well-defined chromatin structure. The central part of the organism often stains less deeply than the periphery, and ring-forms like those of the malaria parasite may be observed. Some species show small rod-shaped forms. Multiplication occurs by binary fission and pairs of individuals partially attached to one another may be seen inside the red cells. For microscopic demonstration, blood films are stained by a Romanowsky stain—*e.g.* Leishman's or Giemsa's. These organisms are usually transmitted by ticks, and the eggs may also become infected from the parent insect. For detailed information regarding the biology and life-cycle of these organisms, one of the works on protozoology should be consulted.

Babesia bigemina is the causative organism of Red Water Fever of cattle in North and South America, Africa and Australia. It is transmitted by ticks (*e.g.* *Margaropus annulatus*). It occurs in characteristic pairs in the red cells. The individuals are pear-shaped with the pointed ends apposed.

Babesia canis is the organism of Biliary Fever of dogs. In

the blood the babesiae are seen both in the red cells and plasma. The intracellular forms are pear-shaped or irregular, and several may be found in one corpuscle. The free forms are rounded.

Babesia (Theileria) parva of East African Coast Fever in cattle appears as a small rod-shaped body inside red cells. Ring-forms may also be observed.

Organisms resembling *Babesia bigemina* occur in Biliary Fever of horses (*Babesia equi*) and in haemoglobinuria of sheep (*Babesia ovis*). A bovine piroplasmosis in South Africa, due to a species resembling, but distinct from, *Babesia bigemina* has also been described (*Babesia mutans*).

A genus designated *Anaplasma*, originally thought to be related to *Babesia*, has been described in Gall Sickness of cattle in South Africa. The organisms, as seen in blood films stained by Giemsa's stain, appear as minute rounded bodies situated near the margin of the red cell (*A. marginale*), in size about one-tenth of the diameter of the cell. They are described as possessing a central granule. These structures may possibly be related to *Bartonella* (*vide p.* 431).

TRYPANOSOMES PATHOGENIC TO MAN

Protozoa of the sub-class Flagellata. Infection with these organisms is designated by the general term, Trypanosomiasis.

Three species are pathogenic to man: *T. gambiense* and *T. rhodesiense* of African Sleeping Sickness, and *T. cruzi* of Brazilian trypanosomiasis. The last-named is sometimes classified in a separate genus, *Schizotrypanum*.

TRYPANOSOMA GAMBIENSE

Biology.—This organism is an elongated, sinuous, fusiform structure, 15–30 μ long by 1.5–3 μ broad, with a longitudinal undulating membrane and a flagellum projecting from one end. It is motile, and in moving the flagellum is anterior. In stained preparations two nuclear structures are noted, the larger or tropho-nucleus situated about the middle of the organism, and the smaller, micro- or kineto-nucleus (or kinetoplast) at the posterior end. The latter stains deeply and is surrounded by an unstained halo. Chromatin-like granules are seen in the protoplasm independently of the nuclei. From the kineto-nucleus arises a filament

(axoneme) which forms the free edge of the undulating membrane and is continued into the flagellum, forming its central core. This structure stains like chromatin. Some morphological variation is noted among individual organisms, some being relatively long and slender with long free flagella, others broader and with a narrow undulating membrane and short flagellum. It has been suggested that these are sexually different, but this is doubtful.

Multiplication is by longitudinal amitotic fission.

Occurrence.—The infection is transmitted by the bite of *Glossina palpalis* (*Tse-tse fly*). It seems probable that the trypanosome undergoes some stage in its life-cycle in the fly. Shortly after blood from an infected person is ingested by the insect, transmission is possible in a mechanical fashion, but in a few days the trypanosomes disappear from the alimentary tract. A later stage of infectivity occurs after about twenty days; the trypanosomes may multiply in the intestine, and pass to the proventriculus, the salivary glands and proboscis. Multiplying in the insect, the trypanosome may show considerable change of form. Thus the flagellum and undulating membrane may be lost or rudimentary and the kinetonucleus becomes anterior to the macronucleus (erithidial form).

It would appear that *T. gambiense* occurs also in certain wild herbivora—e.g. the antelope—from which it is transmitted by tse-tse flies to man.

Two to three weeks after infection a febrile condition develops, and trypanosomes are present in the blood, though not as a rule sufficiently numerous to be readily demonstrable. The superficial lymph glands become enlarged, and trypanosomes can be demonstrated by puncture and aspiration with a syringe. In the advanced stages of the disease, when the characteristic lethargy has developed, the parasites can be detected in the cerebro-spinal fluid.

Animal Inoculation.—Monkeys injected with infective material develop a disease which is more or less similar to human trypanosomiasis.

Guinea-pigs can be infected, and trypanosomes appear in considerable numbers in the blood, but the infection is either unassociated with any obvious pathological condition, or the resulting disease is very chronic in its course.

Cultivation of Trypanosomes.—See p. 119.

TRYPANOSOMA RHODESIENSE

Associated with a more acute form of Sleeping Sickness than that produced by *T. gambiense*.

Morphologically it resembles *T. gambiense*, but, in an inoculated animal, a certain number of the trypanosomes (about 5 per cent.) show the tropho-nucleus situated posteriorly near the kinetonucleus ("posterior nucleated" forms).

This species is also more virulent to laboratory animals.

Glossina morsitans is the insect vector.

It is supposed that this organism is identical with *T. brucei*, the organism of Nagana.

TRYPANOSOMA (or SCHIZOTRYPANUM) CRUZI

The cause of human trypanosomiasis in Brazil.

Its first development after infection occurs in the endothelial and tissue cells of internal organs, in the muscles, and in the heart wall. It is non-flagellate at first, and resembles a *Leishmania* (*vide infra*). It may, however, appear in the blood as a typical flagellate trypanosome.

It is transmitted by a bug *Conorhinus megistus* (syn. *Triatoma megista*).

Various laboratory animals are susceptible to experimental inoculation—e.g. guinea-pigs, white rats, monkeys.

DIAGNOSIS OF TRYPANOSOME INFECTION

In the first place the peripheral blood should be examined. As trypanosomes may be scanty, "thick-films" are prepared and stained by Leishman's stain as in malaria diagnosis (*q.v.*). Fresh preparations of the blood may also be examined microscopically (*vide p. 444*).

A method of concentrating trypanosomes in the

blood has been applied as follows :—5–10 c.c. of blood are withdrawn from a vein into 20 c.c. of 1 per cent. sodium citrate solution, and the mixture is centrifugalised for about ten minutes ; the plasma and leucocyte layer on the surface of the blood sediment are withdrawn and re-centrifugalised ; this is repeated two or three times ; the deposit is examined, in the form of fresh preparations and stained films, after each centrifugalisation.

If superficial lymph glands are enlarged, puncture and aspiration with a syringe may be carried out, and the “ juice ” examined. The syringe should be perfectly dry if this procedure is to be successful.

Blood, gland juice, or an emulsion of an excised gland injected into a guinea-pig may yield a positive diagnosis where other methods fail. The blood of the animal is examined in fresh preparations or stained films at intervals after the inoculation.

In the lethargic state, 10 c.c. of cerebro-spinal fluid are withdrawn, and centrifugalised for fifteen to twenty minutes ; the deposit is then examined either in the form of a fresh preparation under a cover-glass ringed with vaseline, or in stained films.

TRYPANOSOMIASIS OF ANIMALS

Trypanosome infections occur in a variety of animals. Different species of trypanosomes are recognised. These have the general characters of the genus as described in the case of *T. gambiense*, but show variations in certain of their characters.

T. brucei : the organism of Nagana or Tse-tse Fly disease occurring in horses, other equidae, dogs and cattle in Africa, is transmitted by *Glossina morsitans*. It is sluggish in movement and corresponds in its morphology with *T. rhodesiense* (*vide supra*), showing posterior nucleated forms.

T. vivax, so called in virtue of its active movement, and *T. congolense*, produce infections in domesticated animals in Africa and are transmitted by tse-tse flies. *T. vivax* possesses a free flagellum and its macronucleus is anterior. *T. congolense* is a smaller organism (8–19 μ long) and has no free flagellum.

T. equiperdum occurs in horses, producing the disease known as Dourine ; the infection is transmitted by coitus. The organism shows a free flagellum and the nucleus is central.

T. equinum is the organism of Mal de Caderas, a South American disease of horses. A feature of the organism is the inconspicuous kintonucleus which stains feebly.

T. evansi, the organism of Surra affecting domesticated animals (including camels) in various parts of the world. It is actively motile, with a free flagellum and a blunt posterior end. It is conveyed mechanically by *Stomoxys* and *Tabanus* flies.

T. lewisi is an exceedingly common blood parasite of rats, and is world-wide in distribution. It is an actively motile, narrow trypanosome, with a pointed posterior end and anteriorly a free flagellum, the macronucleus being situated about the middle of the organism. The kintonucleus is rod-shaped, and stains deeply. It produces little disturbance in the health of the host. Infection is transmitted by rat fleas.

T. theileri is responsible for an infection occurring in cattle in Africa. It is unusually large ($25-70\mu$ in length). The flagellum is free. It is transmitted by a Tabanid fly.

For further details of the pathogenic trypanosomes one of the larger works on bacteriology or protozoology should be consulted.

THE LEISHMANIAE

These are pathogenic protozoa biologically related to the trypanosomes, but more closely allied to the genus *Herpetomonas*.

They are designated as follows :

L. donovani—of Kala-azar (occurring in certain Eastern countries).

L. tropica—of Tropical Ulcer or Delhi boil. This is generally regarded as a separate species.

“*L. infantum*”—of Infantile Splenomegaly (occurring in Northern Africa). It is doubtful whether this form constitutes a separate species or is related to *L. donovani*.

“*L. braziliensis*”—of Espundia (or Uta), occurring in South America. This disease resembles in some respects Tropical Ulcer.

L. DONOVANI

Biology.—In morphology it is an oval organism about $2.5-3.5\mu$ in its longest diameter.

Stained with a Romanowsky stain two nuclear

structures are observed, one large and rounded (macronucleus), and the other small, deeply staining, and rod-shaped (blepharoplast). The protoplasm may be vacuolated. The organism multiplies by binary fission.

The *Leishmania* is typically intracellular in the tissues, situated in the endothelial cells of the spleen, liver, bone marrow and lymphatic glands. It may also be found in large mononuclear cells in the peripheral blood. One endothelial cell may contain considerable numbers of organisms.

Cultures can be obtained from the spleen on N.N.N. medium (*vide* p. 119) incubated at 20°–24° C. In culture the organisms increase in size and elongate; the blepharoplast becomes situated at one end, and from it a flagellum arises. No undulating membrane develops. Thus *Leishmania* in culture assumes the biological characters of a *Herpetomonas*.

Monkeys and dogs can be infected experimentally.

Transmission of the disease is by insect agency. The evidence points to the sand-fly, *Phlebotomus argentipes* as the vector of infection.

DIAGNOSIS

Thick blood films should be examined as in malaria diagnosis, but in a large proportion of cases it may be impossible to demonstrate *Leishmania* in the peripheral blood.

During life the diagnosis may be established by aspirating fluid from the enlarged spleen by "spleen puncture." A dry needle should be used. This procedure is not without danger to the patient, and for this reason liver puncture has been preferred by some workers, though the likelihood of finding the organism in fluid from the liver is less than in the case of the spleen.

It has also been claimed that a diagnosis may be established by culturing peripheral blood on N.N.N. medium.

L. TROPICA

This organism is similar to *L. donovani* and shows the same intracellular distribution. Besides the characteristic oval forms, elongated organisms may be noted. In culture on N.N.N. medium the herpetomonad forms develop as in the case of *L. donovani*. Monkeys and dogs can be infected experimentally. Transmission is probably by *Phlebotomus pappatasi*.

DIAGNOSIS

Diagnosis.—Films are made from the exudate of the ulcer after carefully cleansing the surface and removing the surface discharge. They are stained by Leishman's stain.

"L. INFANTUM"

This organism is similar to *L. donovani*, and is associated with a pathological condition, similar to that of Kala-azar, occurring in young children in North Africa and the Mediterranean littoral. The infection also occurs naturally in dogs, which are possibly the origin of the human disease, the organism being transmitted by an insect (probably *Phlebotomus perniciosus*). Monkeys, dogs and guinea-pigs are susceptible to experimental infection.

Diagnosis is established as in the case of Kala-azar.

CHAPTER XXIII

FILTERABLE VIRUSES ¹

CONSIDERABLE attention has been paid in recent years to a group of infective agents producing disease in man, animals, insects and plants, which, on account of their minute size, are able to pass through the pores of earthenware and collodion filters.

The question has been much debated whether the filterable viruses are living organisms like the bacteria. All the evidence at present available, whether microscopical or experimental, shows that these infective agents are particulate organisms comparable with, though very much smaller than, the ordinary microbes, and no alternative explanation can be entertained. It has been thought that the viruses differ essentially from the bacteria in respect of three characteristics: (a) ultramicroscopic dimensions, (b) non-cultivability *in vitro*, and (c) filterability. It should be emphasised, however, that none of these criteria really hold, for the "elementary particles or bodies" seen microscopically in certain virus diseases (*vide infra*) actually represent the causative viruses, and Barnard has photographed virus particles in a number of cases by ultra-violet light. Many viruses can be cultivated along with tissue cultures, and when their biological requirements are better known, simpler media will probably be as effective. Moreover, organisms which cannot be classed with the viruses may pass earthenware filters. These facts will be elaborated later but, at the outset, it should not be assumed that

¹ In revising this chapter the authors have to acknowledge their indebtedness, for much of the information cited, to various papers and monographs, particularly the review by Burnet and Andrewes, *Zent. f. Bakt. Orig.*, 1933, vol. 130, p. 161, and *A System of Bacteriology*, Medical Research Council, 1930, vol. vii., which latter should be consulted for further details of the subjects referred to.

there is a distinct biological separation of the viruses and the ordinary bacteria. It may be said that much of our recent knowledge of the filterable viruses has been contributed by British and American workers.

As explained on p. 51, the limit of visibility for individual particles is about 0.25μ when direct illumination by transmitted light is used, and is governed by the wave-length of the light employed. By dark-ground illumination however (*q.v.*) a very minute object may be rendered visible owing to its refraction of light, and is seen as a luminous spot. No resolution is obtained; that is, no details of its form or structure are revealed, but its presence merely is noted. Resolution is determined by the wave-length of the light used, the shorter the wave-length the smaller the object that can be resolved. With ordinary transmitted light the limit is 0.25μ . If, however, short-wave ultra-violet light be used much smaller objects can be resolved, but as the eye is insensitive to such short waves photographic methods must be employed. For this work a special microscope having an optical system constructed entirely of quartz (since glass absorbs ultra-violet rays) must be employed, while the technical difficulties are great. Under these circumstances Barnard has succeeded in resolving and photographing objects 0.075μ in diameter, and in the future it is probable that even smaller particles will be observed, so that the term "ultramicroscopic" is becoming a misnomer.

The evidence regarding viruses which has accumulated during the past few years indicates that those of animal origin are definitely organised living structures comparable with ordinary bacteria, but very much smaller in size. They propagate, infect tissues, incite immunity, etc., like the visible bacteria, and there is no reason why the range of micro-organisms in nature should be bounded by the limit of human vision with ordinary light. Just as the visible pathogenic organisms vary in size from that of the anthrax bacillus down to the influenza bacillus and the pleuro-

pneumonia organism, so there are viruses ranging from the pleuropneumonia organism to a size probably not much larger than a molecule of the more complex proteins; for example, the virus of foot-and-mouth disease is only slightly larger than the molecule of oxyhaemoglobin. The larger viruses can be photographed, while the sizes of the virus particles can fairly accurately be determined by filtration through graded collodion membranes. Immune serum shows specific neutralising properties against the virus concerned, and in certain cases such as herpes and vaccinia the antibodies can be absorbed by suitable virus-material. Comparable with the serological types of various bacteria, such as the pneumococcus or meningococcus, are the three antigenically distinct strains of the foot-and-mouth disease virus termed O, A, and C. Moreover the viruses when transmitted by animal passage, whether in the same or different species, always "breed" true to type and reproduce the same disease.

SIZE OF THE VIRUS PARTICLES

By filtration through thin, collodion membranes certain viruses have been graded according to size (Elford).

The following Table indicates the size of certain filterable viruses ascertained by this means, mostly by Elford and his colleagues.

<i>Virus</i>	<i>Size</i>
Foot-and-mouth disease	8--12 μ ¹
Louping ill	15--20 μ
Yellow fever	18--27 μ
Rift Valley fever	23--35 μ
Fowl plague	60--90 μ
Borna disease	0.085--0.125 μ
Pseudo-rabies	0.1--0.15 μ
Herpes	0.1--0.15 μ
Infectious ectromelia	0.1--0.15 μ
Vaccinia	0.125--0.175 μ
Canary-pox	0.125--0.175 μ

¹ μ = the one-thousandth part of μ = 0.000001 mm.

The sizes of several of these viruses have also been ascertained by ultra-violet photography (Barnard) and the results correspond very closely with those obtained by filtration.

FILTRATION OF THE VIRUSES

As the viruses are distinguished by their capacity to pass through filters which are impervious to ordinary bacteria it is essential to understand the types and limitations of the various filters used. These may be subdivided into (a) coarse filters, (b) fine filters, (c) collodion membranes. The term filterable, though commonly used, may be misleading. It was originally applied in respect to rather coarse earthenware filters of the Berkefeld type which would just hold back the smallest bacteria—e.g. *Bacillus prodigiosus*.¹

Four types of "filterable" organisms can be recognised :

- (1) The viruses.
- (2) Filterable bacteria—e.g. *Bacterium pneumosintes*, and similar anaerobic bacteria of the respiratory passages, which pass coarse filters.
- (3) Filterable forms of bacteria—e.g. of the pleuropneumonia organism and the reported filterable forms of the tubercle bacillus and other visible bacteria.
- (4) Certain spirochaetes which, owing to their extreme slenderness, plasticity and motility, are able to pass through some of the coarser filters.

It is important to note that the mere passage of an organism through an earthenware filter candle does not justify the term "filterable virus" being applied to it.

When describing a virus or organism as filterable it is necessary therefore to indicate (1) the type of filter; (2) the porosity of the filter; (3) the pressure

¹ *B. prodigiosus* is a minute Gram-negative bacillus which, growing on culture medium, produces a red pigment.

employed during filtration, and (4) the controlling organism with which the filter has been tested (*vide infra*).

Filtration is not the mere mechanical passage of particles of a certain size through slightly larger pores, but depends on physico-chemical factors. Thus the earthenware filter consists mainly of magnesium and calcium silicates, and according to the electric charge on the virus, so the particles will be adsorbed or be able to pass through. The more acid the solution the more liable the particles are to enter into combination with the silicate radicle, forming an insoluble compound and becoming adsorbed. In addition, the adsorption of a virus by protein or tissue, the amount of the latter substances in solution, the temperature and pressure of filtration, affect very profoundly the results obtained. Filtration, therefore, depends on many variable and uncontrollable factors, and inability to pass a Berkefeld or Chamberland filter is no measure of size.

It must also be emphasised that, with few exceptions, the presence of virus in the filtrate can be determined only by animal experiment. As certain viruses are very susceptible to changes in pH it is essential that the reaction of the virus-material be carefully adjusted, otherwise the virus may be inactivated and so adjudged non-filterable.

COARSE FILTERS

These are made from Kieselguhr, a diatomaceous earth found in deposits in Germany and other parts of the world. Filters made from this material are coarse—that is, have relatively large pores owing to the size of the granules forming the substance of the filter. The commonest type of coarse filter is the Berkefeld, which is made in three grades of porosity—namely, V (viel) the coarsest, W (wenig) the finest, and N (normal) intermediate. Of these, the Berkefeld V is the one usually employed, and it should not pass a small organism such as *B. prodigiosus*.

A similar type to the Berkefeld is the Mandler filter, manufactured in the United States.

The coarser grades of the Chamberland filters are similar to the Berkefeld types. The most porous, L₁, allows most organisms to pass, being merely a clarifying filter. The next three, L_{1a}, L₂ and L₃, are comparable with the Berkefeld V, N, and W candles respectively.

FINE FILTERS

These are made of unglazed porcelain. The Chamberland (French) is an example and is produced in various grades of porosity. The finer grades will pass only certain ultramicroscopic viruses of extreme minuteness, such as the viruses of foot-and-mouth disease and of fowl plague. The porcelain filters are used extensively for the removal of organisms from fluid cultures in order to obtain the bacterial toxin.

In *Seitz filters* the fluid is passed through a film of an asbestos composition (*vide* p. 73), and various sizes for laboratory work are available. After use the asbestos film is discarded. The films are supplied in three grades—termed coarse, normal, and “special” E.K. These filters are very convenient and useful when bacteria-free filtrates are required, and the larger sizes are especially valuable in the sterilisation of serum for making media. The normal and fine grades of films do not allow the ordinary test bacteria—e.g. *B. prodigiosus*—to pass.

COLLODION FILTERS

Elford¹ has devised a technique for preparing collodion membranes of graded sizes of porosity which he terms *gradocol* membranes, since they are products of graded coagulation of collodion. Collodion films show two types of structure :

- (a) *microgel*, which has a coarse structure visible microscopically, and
- (b) *ultragel*, the structural elements of which are not resolvable by the microscope, but which is built up of particulate matter. Hitherto these membranes, although uniform, have not been sufficiently permeable for filtration work.

¹ For details of technique see W. J. Elford, 1931, *J. Path. and Bact.*, vol. 34, p. 505, and 1933, *Proc. Roy. Soc., B.*, vol. 112, p. 384.

The *gradocol* membranes possess the permeability of the microgel type, but have the ultragel structure and are very uniform in their porosity. They are made from an acetone solution of collodion (nitro-cellulose) diluted with an ethyl alcohol-ether mixture to which are added varying amounts of amyl alcohol. 75 c.c. of the mixture are poured into a shallow cell 20 cm. in diameter in a constant-temperature room (22.5° C.), allowed to evaporate for varying periods of one to three hours, and then washed over an extended period with distilled water. By varying the amount and composition of the collodion mixture, and the conditions of evaporation, permeable filters of average-porosity size (A.P.S.) ranging from 3 μ down to 10 m μ or less have been prepared. It is possible to reproduce accurately at any time filters of any desired permeability and porosity. The technical details are elaborate and of extreme importance.

By means of these collodion membranes it has been possible to determine the size of many of the viruses.

Methods of filtration are dealt with in the appendix to this chapter, p. 483.

When working with filterable viruses, controls must always be made of the filtered material. It must be cultivated both aerobically and anaerobically to show that no ordinary bacteria are present. Inoculation of animals susceptible to the virus should be carried out, and the filtrate should give rise to the train of symptoms characteristic of the disease.

HUMAN DISEASES IN WHICH THERE IS EVIDENCE THAT THE CAUSAL AGENT IS A FILTERABLE VIRUS

Smallpox (*variola*), *alastrim* and *vaccinia*.

Chickenpox (*varicella*).

Rabies (common also to dogs and cattle).

Epidemic poliomyelitis.

Herpes febrilis.

Yellow fever.

Rift Valley fever (common also to sheep and cattle).

Psittacosis (common also to parrots).

Measles.

Molluscum contagiosum.

Common warts.
Mumps.
Influenza.
Common cold.
Phlebotomus fever.
Dengue.
Climatic bubo, or lymphogranuloma inguinale.

The following Human Diseases are thought to be due to Filterable Viruses, but the evidence is not conclusive :

Rubella (German measles).
Encephalitis lethargica.
Herpes zoster.
Trachoma (a visible bacterium has also been described by Noguchi as the causal agent, but this has not been confirmed).

The more important Diseases of Animals generally accepted as due to Filterable Viruses :

Foot-and-mouth disease (sometimes transmitted to man).
Canine distemper.
Feline distemper.
Sheep-pox and cowpox (vaccinia—*vide supra*).
Swine fever (or hog-cholera).
Cattle plague (rinderpest).
Vesicular stomatitis of horses.
African horse sickness.
Borna disease of horses.
Infectious anaemia of horses.
Rabies (*vide supra*).
Pseudo-rabies or Mad Itch of cattle.
Louping ill of sheep (sometimes affects man).
Blue-tongue of sheep.
Nairobi sheep disease.
Encephalitis of silver foxes.
Swine influenza.
Virus myxomatosum of rabbits.
Filterable fibroma of testis of rabbits.
Salivary-gland disease of guinea-pigs.
Infectious ectromelia of mice.
Virus III of rabbits.

(Pleuropneumonia of cattle has been classified as a filterable virus disease, but the organism is within the range of microscopic visibility and must be considered as a bacterium)

Diseases of Birds :

- Filterable tumours of fowls—*e.g.* Rous sarcoma.
- Fowl plague.
- Fowl-pox, including roup.
- Pigeon-pox.
- Leukaemia of fowls.
- Canary-pox
- Psittacosis (*vide supra*).
- Pacheco's parrot disease (*not* transmissible to man—*cp.* psittacosis).

Diseases of Insects :

- Silkworm jaundice.
- Sac brood of bees.

Diseases of Plants :

- Mosaic disease of tobacco and tomato plants, and diseases affecting almost all plants.

Note.—The bacteriophage, described briefly on p. 23, also shows many analogies with the viruses, and has been regarded by some workers as a virus parasitic towards the bacteria.

GENERAL CHARACTERS OF THE FILTERABLE VIRUSES

The filterable viruses differ from the ordinary bacteria in many ways, and certain characteristic features are common to many of the series :—

1. Great infectiousness of the associated disease.
2. The production of immunity, which may be of long or short duration.
3. Filterability.
4. Invisibility.
5. Non-cultivability *in vitro* by ordinary methods applicable to bacteria.
6. Resistance to glycerol.
7. The presence of “*inclusion bodies*”^{¶¶} (or “*cell inclusions*”) in lesions.

1. GREAT INFECTIOUSNESS OF THE ASSOCIATED DISEASE.—This is seen in smallpox epidemics, where, in spite of careful quarantine, cases continue to occur.

Foot-and-mouth disease is another example; the difficulty of control is due to the amazing rapidity of spread, not only from animal to animal, but from one locality to another.

As a group, these viruses are markedly pathogenic, even in minute doses. The virus of yellow fever is capable of causing infection of the monkey in a quantity as small as 0.000001 c.c. of an emulsion of brain tissue from an infected animal. Incredibly small amounts of these viruses are capable of causing infection, as is shown by the rapid spread of the disease in spite of the utmost attempts at control.

2. THE PRODUCTION OF IMMUNITY.—In individuals surviving infection there is in many of the diseases a high degree of immunity, which usually lasts for long periods of time. Advantage is taken of this in immunisation against smallpox and rabies by inoculation with attenuated viruses. In addition, specific antisera may be prepared, as in the case of hog-cholera and cattle plague, which are available for specific treatment. On the other hand, the immunity following infection may be of only short duration.

Prolonged Immunity

Smallpox.
Vaccinia.
Chickenpox.
Measles.
Yellow fever.
Rift Valley fever.
Mumps.

Short Immunity

Herpes febrilis.
Influenza.
Common cold.
Dengue.

Generally speaking, immunity cannot be conferred by injecting dead virus into susceptible animals as is the case with killed culture vaccines of bacteria, and it has been thought that living virus is absolutely essential for an immunity response. It has been shown, however, that formolised or carbolised virus can be used to produce active immunity against foot-and-mouth disease, rabies, rinderpest, etc.

3. FILTERABILITY.—This aspect has been previ-

ously referred to; all the true viruses will pass collodion membranes.

4. INVISIBILITY.—Organisms smaller than 0.25μ are beyond the limits of visibility by ordinary visual light. In stained preparations the “elementary bodies” of certain diseases are actually the virus, and in consequence these viruses are not truly invisible. Similarly by photography with ultra-violet light certain viruses have been rendered “visible.”

5. CULTIVATION.—The filterable viruses are not cultivable on ordinary media, and their presence is only recognised by their action on susceptible animals. Recently it has been shown that a number of viruses can be grown in association with the proliferating cells of tissue cultures. Simplification of the technique was made by H. B. and M. C. Maitland, who obtained good growths of vaccinia virus, using fresh minced adult hen or rabbit kidney mixed with fresh serum and Tyrode's solution.¹ By a similar technique, many viruses have been cultivated, but the conditions necessary for growth are not yet understood, and the procedure is somewhat empirical. It has been claimed that vaccinia virus can be cultivated in a cell-free medium, but this has not been confirmed. Where there is evidence that the virus can exist only in an intracellular position, as in psittacosis and fowl-pox, an intracellular environment is essential for growth *in vitro*. Up to the present, none of the true filterable viruses have shown colony formation on solid media, which would prove conclusively that the viruses are definite organisms similar to the ordinary bacteria, though on a much smaller scale. It is possible that the strict intracellular parasitism of many of the viruses preclude the formation of definite colonies, as in the case of other intracellular parasites—*e.g.* the plasmodium of malaria.

¹ Tyrode's solution.—NaCl, 8.0 gm., KCl, 0.2 gm., CaCl₂, 0.2 gm., MgCl₂, 0.2 gm., NaHCO₃, 1.0 gm., glucose, 1.0 gm., NaH₂PO₄, 0.05 gm., distilled water, 1000 c.c. The solution is sterilised by filtration through a Seitz filter.

Several facts emerge from the mass of literature on the subject :

(1) Certain filterable viruses can undoubtedly be cultivated *in vitro*.

(2) Different conditions of growth are necessary, and these must be provided just as for the ordinary bacteria, whose oxygen and temperature requirements, accessory growth factors (*e.g.* for the haemophilic organisms), optimum H-ion concentrations, etc., must be studied. Thus, in the case of the common cold virus, anaerobic conditions in living tissues appear to be necessary ; the viruses of herpes and vaccinia multiply in disintegrating or surviving tissue and active cell proliferation is not essential, while the fowl-pox, psittacosis and fowl-plague viruses require actively growing tissue cells. Certain viruses grow irrespective of the nature of the tissue cultures, but the viruses of foot-and-mouth disease and fowl-pox require definite tissues from certain species—*e.g.* the pads, lips, tongue, or hairy skin of the embryo guinea-pig are necessary for the growth of the virus of foot-and-mouth disease.

Intermediate between the ordinary bacteria and the true viruses are the causal organisms of pleuro-pneumonia of cattle and agalactia (p. 481). These were formerly included amongst the filterable viruses, as they pass filters which hold back ordinary organisms. On the other hand, cultures can readily be obtained on serum-agar, which, when stained by Giemsa's method, reveal a great polymorphism of the organism at different stages of growth.

Certain viruses—*e.g.* of fowl-pox and canary-pox—can be cultivated in the cells of the chorio-allantoic membrane of the developing egg of the hen. Characteristic lesions are produced in which there are large amounts of active virus.

6. RESISTANCE TO GLYCEROL. — Ordinary non-spore-forming bacteria are killed by 50 per cent. glycerol in a comparatively short time. Many of the filterable viruses, on the contrary, retain their viability much longer in this material than in any other fluid when kept at 4° C. The preservation of the vaccine virus used prophylactically against smallpox is accomplished by this means. Other viruses which keep for prolonged periods in glycerol at 4° C. are: poliomyelitis virus, the virus of febrile herpes (which we have kept virulent in glycerol for more than one year), and the virus of rabies. On the other hand, some viruses—*e.g.* Virus III

of rabbits—will survive for less time than] certain ordinary bacteria. It is essential that the glycerol be pure and free from mineral acids and sulphates.

(The filterable viruses are very readily destroyed by heat and antiseptics, but may survive outside the body for considerable periods of time, and can also resist desiccation.)

7. When lesions occurring in filterable virus diseases are examined histologically there are frequently found within the cell nucleus, or the cytoplasm, peculiar structures which may be of two types—namely :

- (1) “Elementary bodies”;
- (2) “Inclusion bodies.”

“Elementary bodies” appear to represent the actual virus, and these are exemplified by the “Paschen corpuscles” which are seen in smallpox and vaccinia lesions and which may be demonstrated in the vesicle fluid of early vaccinia in calves. They are small round coccus-like bodies, about 0.25μ in size. Similar bodies have been found in varicella, while they are demonstrable in the lesions of psittacosis, molluscum contagiosum, ectromelia and fowl-pox. They can be demonstrated by Castaneda’s stain (p. 176) and by prolonged staining in Giemsa’s solution, when they appear red or purplish. By comparison with photographs taken with ultra-violet light (Barnard), the effect of staining is to make them appear larger. The work of Ledingham on the Paschen corpuscles of vaccinia, and of Bedson on the elementary bodies of psittacosis, indicate that these bodies represent actual virus particles.

“Inclusion bodies” may be :

- (a) Cytoplasmic—*e.g.* the Negri bodies which are found in the ganglion cells of the central nervous system in rabies, the Guarnieri bodies of smallpox and the Bollinger bodies of fowl-pox. These inclusion bodies are acidophile in nature, and with Mann’s methyl-blue-cosin¹ or

¹ See H. J. Conn, *Biological Stains*, 2nd edit., 1929, p. 112,

Giemsa's stain are coloured pink by the eosin. The Negri bodies are round or oval, and vary considerably in size (*vide* p. 468). Guarnieri bodies are found in the cytoplasm of epithelial cells of the skin lesions of smallpox and vaccinia. They stain with acid dyes and are seen lying in clear spaces in the protoplasm. They may vary in size from 1μ to $15-20\mu$, and may be as large as the cell nucleus itself. Similarly Bollinger bodies are pink-staining, cytoplasmic inclusion bodies, round or oval in shape, and varying considerably in size.

- (b) Intranuclear inclusions, which can be demonstrated by Giemsa's stain, polychrome methylene blue and eosin, and Mann's stain, in the following diseases :

Yellow fever, in the liver. ✓

Herpes, in the epithelial cells.

Rift Valley fever, in the liver.

Pseudo-rabies, in the brain.

Pacheco's parrot disease, in the liver.

Guinea-pig salivary-gland disease, in the cells of the ducts.

Encephalitis of silver foxes, in the endothelial cells.

These intranuclear inclusions are acidophile in nature and are stained reddish or pink, and vary considerably in size.

It has been shown that a single isolated Bollinger body (of fowl-pox) is infective for the fowl and therefore contains the virus. As however this inclusion body is comparatively large, and the virus of fowl-pox passes a filter and is only $60-90m\mu$ in size, the Bollinger body does not indicate the actual form of the virus. When suitably stained and examined microscopically the Bollinger bodies are seen to consist of minute particles—termed Borrel bodies—in an amorphous matrix. These Borrel bodies are similar to the elementary bodies of psittacosis and the Paschen bodies of variola and vaccinia, and are regarded as the virus. In fowl-pox therefore it would appear that the inclusion bodies consist of aggregates or colonies of the virus which is multiplying as an intracellular parasite.

The inclusion bodies were assumed to be a mantle or sheath

around the infective agents, and were formerly known as "chlamydozoa," and while some inclusion bodies may represent degenerative or reactionary changes in the cell due to the action of the virus, recent evidence suggests that they are aggregates or collections of elementary bodies which cannot be resolved by present methods of staining and microscopy.

When a virus has an affinity for nervous tissues it is referred to as *neurotropic*.

POLIOMYELITIS

Poliomyelitis is an acute infection occurring chiefly in children, which affects the central nervous system, with usually a special localisation in the anterior horn of the spinal cord.

The virus can pass through Berkefeld V and N filters.

This virus is easily destroyed by heat, but can withstand the action of weak phenol for some days. In brain tissue, preserved in 50 per cent. glycerol at 4° C., it remains viable for years.

Experimental Inoculation.—The disease can be transmitted to monkeys by the intracerebral or intravenous inoculation of human brain or spinal-cord material. The animals develop the typical disease, and show in the central nervous system histological lesions similar to human cases. The disease can also be transmitted from monkey to monkey.

Infection probably takes place through the nose to the olfactory lobes of the brain. The virus gains direct access to the ends of the olfactory nerves (which are exposed, and not under the mucosa) and travels along the nerve fibres and not by the perineural lymphatics. The disease is spread by carriers, who harbour the virus in the naso-pharynx.

The serum of recovered patients contains antibodies which neutralise the virus, and convalescent serum may be used as a prophylactic, but its value in the treatment of the preparalytic or early paralytic stages of the disease is doubtful.

RABIES

This disease is communicated to the human subject by the bite of a rabid dog or other animal, the infective virus being present in the saliva of the animal. When a person is bitten by a dog suffering from rabies, prophylactic treatment by the Pasteur method or a modification must be carried out without delay, and where there is any doubt as to the condition of the dog, an accurate diagnosis is essential. The animal should be killed, and if the laboratory is at some distance, the head is removed and forwarded in ice. In the laboratory the scalp is reflected, the skull is opened by means of sterile bone forceps and the brain removed with aseptic precautions. The hippocampus, which forms the floor of the lateral ventricle, is dissected out, smears are made by squeezing a portion of the tissue between two slides, and pieces are also fixed for histological examination. In addition, an emulsion is prepared for animal inoculation. The smears are fixed in methyl alcohol for five minutes, and stained by Leishman's or Giemsa's method (*vide* pp. 168, 170) or by Mann's stain. For sections, the tissue is fixed in formalin (*vide* p. 178) and stained as above.

The diagnosis depends on the finding of the characteristic *Negri bodies* in the cytoplasm of the nerve cells. They consist of round, oval or angular bodies, varying in size from $0.5-25\mu$, staining pink with Leishman's, Mann's or Giemsa's stain. The nature of these structures is still a matter of uncertainty, though their occurrence is a specific feature of rabies.

The virus is localised in the nervous tissues and salivary glands, and is transmitted through the body by the nerve fibres only. The incubation period in man is usually from thirty to sixty days. The virus becomes enhanced in virulence after repeated passage in the rabbit, until it kills regularly in six to seven days, and then becomes the "fixed" virus of Pasteur. The virus is easily destroyed by heat and chemicals, but is resistant to glycerol. Carbolic acid attenuates

and later kills the virus, and phenolised suspensions of infected rabbit's brain and spinal cord are injected intramuscularly to produce immunity in persons bitten by a rabid dog. The original Pasteur treatment was to inject infected rabbit's spinal cord, the virulence of which had been reduced by drying. The first injection was an emulsion of cord dried for twelve days, and subsequent daily injections of progressively more virulent nervous tissue were given. This live vaccine has been largely superseded by the phenolised virus described above.

Animal inoculation is carried out as follows :—

A portion of the brain tissue is ground up in a sterile mortar with sterile saline solution, and a rabbit is inoculated intracerebrally with 0.25 c.c. of the emulsion. The head of the rabbit is shaved and sterilised with tincture of iodine. The animal is then anaesthetised with ether, and an incision is made in the skin midway between one eye and the ridge in the middle line at the junction of the parietal and occipital bones. The skull is pierced with a small trephine and the material is injected, using a short ($\frac{1}{4}$ -in.) needle fitted to a 1 c.c. syringe. If the rabies virus is present, the animal becomes paretic in from seven to twenty-three days, and death occurs in fifteen to twenty-eight days. Negri bodies can also be demonstrated in smears or sections from the brain.

OTHER DISEASES OF MAN DUE TO NEUROTROPIC VIRUSES

Herpes febrilis.—If the clear fluid from a herpes vesicle be inoculated on to the scarified cornea of a rabbit, there follows a severe keratitis which can be propagated indefinitely through a series of animals. Some of the rabbits show cerebral symptoms and later die of encephalitis. Herpes virus is present uncontaminated by other organisms in the brain material. It is very infective for rabbits, and recovered animals show a marked immunity.

The virus is filterable through Berkefeld and coarse Chamberland filters. Its size is 0.1–0.15 μ . It can be cultivated easily in minced rabbit testis with rabbit plasma and Tyrode's solution. Even after prolonged culture the virus retains its neurotropic properties.

Typical "inclusions" can be demonstrated in the cells of the culture. In the corneal and nerve cells of infected rabbits, inclusion bodies may be demonstrated. These are acidophile intranuclear bodies, at first small, but later they coalesce and occupy the greater portion of the nucleus. They are similar to the inclusion bodies in other diseases.

(*Herpes genitalis* is due to a virus similar to that of herpes febrilis.)

Herpes zoster is aetiologically distinct from herpes febrilis and the causal agent is identical with or very closely allied to the virus of varicella (*q.v.*).

Encephalitis lethargica.—Little is known of the virus of this disease except that it has on occasions been transmitted to rabbits and monkeys. It is probably allied to the herpes virus. Much confusion has arisen in experimental work on this disease when rabbits have been used, because these animals suffer naturally from a spontaneous meningo-encephalitis, and this condition has been mistaken for the experimental disease.

In a recent epidemic of encephalitis at St Louis, U.S.A., a virus has been isolated which is transmissible to monkeys and mice by intracerebral inoculation.

VARIOLA (SMALLPOX) AND VACCINIA (COWPOX)

Variola or *smallpox* is due to a filterable virus, although filtration experiments may not always be successful owing to the adsorption of the virus by the filter candle and protein material in the suspension. In the skin lesions, inclusion bodies, "Guarnieri bodies," may be seen (*vide* p. 466), and also minute granules, "Paschen bodies."

The virus of smallpox does not reproduce the disease in the ordinary laboratory animals, but only a slight local lesion. In the monkey, however, material from a smallpox pustule, when inoculated intracutaneously, may produce a typical localised "pock," though not a general eruption.

Active immunity is produced by an attack of the disease, or by vaccination with vaccinia virus.

Vaccinia or *corepox* is closely allied to smallpox. The virus is not usually filterable. Lesions are readily produced in calves and rabbits. "Vaccination" in human beings is the application of vaccinia virus to the scarified skin, with subsequent development of a pustule or "pock."

The inoculation of rabbits or calves with the virus after scarification of the skin is followed by a red swelling on the third day, which later becomes papular. By the fifth day the papules have developed into vesicles filled with clear fluid, which soon becomes cloudy and pustular. About a week after inoculation scabbing occurs. Such reactions are obtained even with a high dilution of calf-lymph—*e.g.* from 1 : 1000 up to even 1 : 100,000. If the virus be injected intravenously in large amounts into the rabbit, generalised lesions occur not only over the external epidermis, but in the internal organs. In the epithelial cells after cutaneous inoculation of the rabbit with the virus, large intracellular "inclusion bodies," known as "Guarnieri bodies," may be seen, and in the early stages enormous numbers of very minute bodies, known as "Paschen bodies," about 0.25μ in size.

The Paschen bodies (or corpuscles) have been purified by Ledingham, and they can be agglutinated by a specific antiserum from recovered animals, while complement-fixation tests can be carried out with the purified corpuscles as antigen. These tests can be used diagnostically. The Paschen bodies are now regarded as the actual virus.

Cultivation.—The virus of vaccinia has been successfully cultured, first in a tissue culture and later by a simplified method introduced by H. B. and M. C. Maitland. The medium consists of minced fresh adult kidney, either from a hen or a rabbit, Tyrode's solution and fresh serum. Bacteria-free vaccinia virus is mixed with 0.3 c.c. fresh minced kidney tissue, 3 c.c. fresh rabbit serum added, and the whole diluted to 10 c.c. with Tyrode's solution. The mixture is distributed in 2 c.c. amounts into Carrel flasks and incubated at 37° C. The kidney cells do not proliferate but remain alive for five to

six days, and in association with them multiplication of the virus takes place. Rivers, using a simple medium of chick embryo and Tyrode's solution, has cultivated the virus for use in vaccination against smallpox. Elementary bodies (Paschen bodies) have been demonstrated in cultures.

Calf-lymph for vaccination is made by inoculating the scarified shaved skin of the calf with pustular material from the rabbit. (The virus is inoculated alternately into calves and rabbits.) About the fifth day later, when the lesions are pustular, the scarified area is scraped with a spoon, and mixed in a mortar with glycerol-saline. After storage in the cold for some time, the number of extraneous organisms rapidly diminishes, and the resultant product which contains the virus is called calf-lymph.

Vaccination with vaccinia virus in the human subject produces a localised pustule and confers immunity against variola (smallpox).

POST-VACCINAL ENCEPHALITIS

Within a fortnight of vaccination an acute disseminated encephalomyelitis may supervene in a very small percentage of cases. A disease identical in its clinical and histological characters has been recorded following other infective diseases, such as smallpox and measles, and occasionally in non-exanthematous cases.

Clinically there is paralysis, at first flaccid and later spastic, while meningeal symptoms are frequently noted in children. Histologically the outstanding characteristic is the demyelination of the areas round the blood vessels. This is noted particularly in the white matter of the cerebrum, mid-brain, and pons, and also in the anterior horns of the cord. Congestion and infiltration with mononuclear cells are also present.

It was originally thought that the encephalitis was actually due to the vaccinia virus affecting the central nervous tissue, but it is now considered that post-vaccinal encephalitis is an entirely different disease, due to a separate virus, and is induced by the antecedent disease. (No other virus, however, has been isolated from post-vaccinal encephalitis.) Either the vaccinia infection lowers the resistance of the patient, thus facilitating the entrance of the encephalomyelitis virus to the nervous tissue, or the antecedent disease may alter the virus so that its virulence for nervous tissue is raised.

VARICELLA

Varicella (chickenpox) also is due to a filterable virus. In the tissues minute bodies similar to Paschen bodies can be demonstrated. There is a close association between the virus of chickenpox and that of herpes zoster. Cases of herpes have been followed by outbreaks of chickenpox. Recent immunity experiments (complement-fixation tests) have shown a pronounced cross-immunity between the viruses, which are now regarded as being identical, or very closely allied.

MEASLES

Little is known about the causal agent of measles. The disease is not communicable to ordinary laboratory animals, but it has been claimed that under favourable circumstances a modified form of measles may be produced in the monkey by inoculation with blood taken at the height of the disease. The experimental condition has been transmitted through a series of animals and the virus shown to be filterable.

Serum from convalescent patients has marked protective powers, and if injected in amounts of 5-10 c.c. before the fifth day after exposure to infection will effectively prevent the disease. If given after this time, the disease is modified and the risk of complications considerably reduced.

COMMON COLD

The causative agent of the common cold is a filterable virus, and can be transmitted to man and to chimpanzees. Other animals are not susceptible. By passage through persons its virulence is raised so that colds of unusual severity can regularly be produced. The virus has been cultivated in a medium of minced ten-days'-old chick embryo and Tyrode's solution. Cultures were obtained by concentrating nasal washings from a case of enhanced virulence by vacuum distillation. This inoculum was added to the medium,

covered by a vaseline seal and incubated at 37° C. for four to nine days. A series of subcultures was made, and infection of human volunteers was obtained with material from the twenty-seventh subculture. These results require confirmation.

INFLUENZA

Recently it has been claimed that influenza is due to a filterable virus and that ferrets are susceptible to the disease.¹ Filtrates from nasal washings of influenza patients, when instilled intranasally into ferrets, produced a characteristic train of symptoms. After forty-eight hours the temperature rose, the animal became out-of-sorts, and on the third day catarrhal symptoms appeared. The eyes became watery and there was a variable amount of watery discharge from the nose. The disease lasted for a few days only and was not fatal. It could be transmitted serially in ferrets either by contact or by intranasal instillation of virus material. No other method of inoculation induced the disease. The condition has been maintained for twenty-six passages with one of the strains. The virus could be found only in the nasal mucous membrane of the animal and not in blood, lymph glands or spleen. By filtration through gradocol membranes it has been estimated that the size of the virus is about the same as that of vaccinia or herpes—namely, 0·1–0·175 μ .

The disease could be induced in ferrets only with material from acute influenza cases, and not from normal people or persons with common colds. Serum from recovered animals neutralised the virus, while human sera, particularly from influenza convalescents, neutralised the ferret virus.

This virus is very closely related to the virus of swine influenza, and there is definite cross-immunity between them. It should be noted that in swine influenza two separate agents can be found—namely, the virus, and *B. influenzae (suis)*. The virus alone produces a mild almost unrecognisable disease, while the bacillus by itself is harmless. In natural swine

¹ Wilson Smith, Andrewes and Laidlaw, 1933, *Lancet*, ii, p. 66.

influenza the two agents act together, the virus being the essential factor. When the virus of swine influenza is inoculated in the ferret, however, the disease is not modified by simultaneous inoculation of *B. influenzae (suís)*.

YELLOW FEVER

The infection is spread naturally by the bite of a mosquito, *Aedes aegypti* (*Stegomyia fasciata*), and possibly by other mosquitoes. The disease can be communicated to *rhesus* monkeys either through the bites of infective mosquitoes or from emulsions of internal organs—*e.g.* the liver—from fatal cases. The virus will pass fine filters, its size being 18–27 m μ . It is present in the blood and organs, particularly in the liver. The serum of convalescent patients has strong protective properties.

The virus can be transmitted to the brains of mice, where it produces an encephalitis, and after twenty or more passages becomes “fixed” (with neurotropic properties). This virus will produce encephalitis in monkeys and some other rodents, such as guinea-pigs, field voles, squirrels, etc. The neurotropic virus leads to the production of immune bodies which protect against the viscerotropic virus, and a method of immunising with neurotropic virus *plus* human immune yellow fever serum is now being used for prophylactic vaccination in man.

PSITTACOSIS

This is an epizootic disease of parrots which may affect human beings, often with fatal results. The virus is present in the organs of fatal cases of both birds and man, and can be transmitted to parrots, budgerigars, guinea-pigs, mice and monkeys, and has a fair resistance to glycerol. It is filterable through Chamberland L₁, L₂, and Seitz E.K. filters. In the cells of the spleen and other organs minute “elementary bodies” can easily be seen after staining by Castaneda’s stain (p. 176). They are round or slightly oval in shape, bright blue in colour, as compared with the pink cells and background, and are found inside reticulo-endothelial cells which are sometimes filled to bursting-point. These “elementary bodies” can be centrifuged and obtained free of cellular material, and are agglutinated specifically by an anti-psittacosis serum, and with it fix complement. The available evidence indicates that these bodies are actually the virus.

In tissue culture of mouse spleen the virus multiplies in the cells. Bedson has described a life-cycle in the development of the virus in which the elementary bodies develop into amoeboid forms which coalesce and form a homogeneous plaque or plasmodium. The plaque then divides up into a number of portions of equal size, oval in shape, so that the virus is represented by spherical bodies filled with oval segments. These segments divide and subdivide, become smaller and more basophile, until eventually typical elementary bodies are formed again.

A somewhat similar infection is Pacheco's disease of parrots, which differs from psittacosis in not being transmissible to man. In the livers of infected parrots numerous inclusion bodies are to be seen.

MUMPS

The infection is transmitted by the saliva. Injection of the saliva from early cases of mumps into the salivary duct of monkeys leads after an incubation period of from six to eight days to a rise in temperature, swelling and oedema of the salivary glands and a lymphopenia. The disease can be transmitted in series by means of infective parotid gland. The virus is filterable through Berkefeld filters, resists desiccation and is preserved by 50 per cent. glycerol. It is neutralised by serum from recovered cases of mumps.

CLIMATIC BUBO or LYMPHOGRANULOMA INGUINALE

This disease is transmitted by venereal contact. It is increasing in frequency both in this country and on the Continent. The appearance for a few days of a small primary sore on the external genitals is followed in males by a swelling of the lymph nodes in the groin; in females where the primary sore is often situated in the vagina the inflammatory reaction may affect the peri-anal tissues, leading to stricture of the rectum and elephantiasis of the genitals. The virus inoculated into the groins of guinea-pigs, monkeys and dogs produces a bubo; inoculated intracerebrally into monkeys, mice, guinea-pigs, dogs and cats, it gives rise to a fatal meningo-encephalitis. Serum from recovered cases is capable of neutralising the virus. A saline extract of infected lymph nodes heated to 60° C. when inoculated intradermally in persons who have suffered from climatic bubo leads to a specific inflammatory reaction in the skin (Frei's reaction).

CANINE DISTEMPER

Distemper is a specific infectious disease of great prevalence in young dogs and other animals, such as ferrets, silver foxes and fitches. The common manifestations are pyrexia and coryza, followed by secondary inflammation of the respiratory system. Experimental evidence goes to show that the infective agent is a filterable ultramicroscopic virus. The disease can be transmitted experimentally to dogs and ferrets by subcutaneous inoculation with discharges from infected animals, and also with blood, serum and certain tissues: e.g. spleen. Filtrates of infective material are also virulent.

Active immunisation of dogs as a prophylactic against distemper can be achieved by two methods:

(1) The use of "vaccine" followed by "virus."

The "vaccine" is formalised liver from dogs having distemper, while the "virus" is a liver extract from ferrets inoculated with distemper virus. Amounts of 5 c.c. of vaccine are given subcutaneously, followed seven to twenty-one days later by 1 c.c. of virus subcutaneously, or 0.25 c.c. virus intradermally.

(2) By injecting immune serum *plus* virus.

The dog to be immunised receives 10 c.c. of immune serum, and at the same time is given 1 c.c. of the virus subcutaneously. The serum prevents the active disease.

The results of these methods have been highly satisfactory in the prophylaxis of distemper in dogs.

B. bronchisepticus and other bacteria, at one time described as the causal agents of distemper, are secondary invaders.

Similarly cat distemper, which affects all the Felidae, is due to a virus followed by secondary invading bacteria.

FOOT-AND-MOUTH DISEASE

Foot-and-mouth disease is an extremely infectious epizootic condition of cattle, and certain other animals, characterised by the occurrence of vesicles on the feet and in the mouth, along with constitutional symptoms of infection. It is occasionally communicated to man by contact and through the agency of milk. The disease can be transmitted experimentally by the inoculation of fluid from the vesicles even after filtration through the finest earthenware filters. No visible or cultivable organisms have been found aetiologically associated with the disease, and the infection is due to a filterable virus. The virus is present also in the saliva, milk and excreta of infected animals, and it is supposed that recovered animals may carry the infection for considerable periods. The

size of the virus, which is the smallest yet recorded, is 8–12m μ . It has been cultivated in tissue culture by using the pads, lips, tongue and hairy skin of embryo guinea-pigs. The disease can be produced experimentally in guinea-pigs by cutaneous inoculation in the hairless pads of the feet. Within four days pyrexia results, and an inflammatory condition of the foot, with a vesicular eruption like that of the natural disease. Secondary vesicles occur on the other feet and in the mouth. The animal usually recovers and is thereafter immune to further infection. The virus is present in the circulating blood as well as in the vesicular lesions. If slowly dried the virus retains its viability for considerable periods and the disease may be transmitted by certain vehicles of infection—e.g. fodder—as well as by contact. It is highly susceptible to heat, and is rapidly killed at 55° C. Three immunological types of the virus have been recognised—namely, O, A, and C. Animals recovered from one type are not immune against another type, although a slight degree of cross-immunity does exist. Active artificial immunity can be obtained by the injection of formalised virus. Immunity following recovery is apparently due to a viricidal antibody present in the blood serum.

RIFT VALLEY FEVER

This disease, also known as enzootic hepatitis, was first described by Daubney and Hudson in 1931 in Kenya Colony, British East Africa, where it caused an extremely fatal epizootic amongst lambs. The ewes also were affected, but not so fatally. The native shepherds and Europeans engaged in the epizootic developed a dengue-like fever with severe back pains, while laboratory workers also become infected. The virus of Rift Valley Fever will pass fine Chamberland filters, and by means of collodion membranes its size has been determined as 23–35 m μ . It has been cultivated in tissue cultures. In addition to being infective for sheep and man, the disease can be transmitted by blood, liver, or spleen tissue to cattle, monkeys, and rodents such as rats, mice, field voles, dormice and squirrels. It is not infective for birds, reptiles, rabbits, or guinea-pigs. In the natural disease in the sheep there is extreme necrosis of the liver, with haemorrhages into other organs. Inclusion bodies are numerous in the liver. The serum of recovered animals neutralises the virus. The virus is resistant to phenol, and in defibrinated blood containing 0.5 per cent. phenol will retain its activity for many months in the cold. The disease is not contagious and there is evidence to suggest that it is transmitted by a mosquito, probably *Taeniorhynchus brevipalpis*.

LOUPING ILL

This disease is an encephalomyelitis of sheep characterised by cerebellar ataxia and disorder of nervous functions. It is common in certain parts of Scotland. The virus is readily filterable through Berkefeld and coarse Chamberland filters. Its size has been estimated as 15–20 μ . The virus can be transmitted by intracerebral injection of emulsions of infected brain and spinal cord to sheep, pigs and mice, and in the latter animal also by intranasal instillation. The evidence points to the tick, *Ixodes ricinus*, as the vector of the disease.

BORNA DISEASE OF HORSES

This is an infectious meningo-encephalomyelitis of horses, characterised by lesions in the central and peripheral nervous systems. After general systemic disturbances the animal becomes paralytic, and usually dies, the mortality being at least 75–80 per cent. The virus is essentially neurotropic. It may also infect cattle and sheep under natural conditions. It can be transmitted experimentally by intracerebral inoculation to these animals, and also to rabbits, guinea-pigs, rats, mice, fowls and monkeys. The virus can pass Berkefeld and the coarse and medium grades of Chamberland filters. Its size is computed as 0.085–0.125 μ and it has not yet been cultivated. The microscopic lesions in the brain are perivascular and meningeal infiltrations with mononuclear cells, with also some diffuse infiltration. Similar lesions occur in the cord. The nuclei of the nerve cells contain characteristic inclusion bodies known as Joest-Degen corpuscles, and are more numerous in the cells of the spinal ganglia. They vary in size from 3 or 4 μ down to the limit of resolution, and by Mann's methyl blue cosin stain are coloured red. The larger bodies are similar to the Negri bodies of rabies. The virus material treated with carbolic acid and glycerol possesses immunising properties.

RINDERPEST

Rinderpest or cattle plague is characterised by elevation of temperature, glairy discharge from eyes and nose, abdominal pains and severe diarrhoea, followed by weakness and death, usually in four to seven days. The causative organism is a filterable virus which passes Berkefeld and coarse Chamberland filters. The virus is not free in the blood stream but is present only in the leucocytes. It is rapidly destroyed by drying, by heat, by chemicals, and if kept at room temperature. It survives for some months if infected blood is frozen solid,

but is rapidly destroyed by glycerol. The virus is present in the discharges, all of which are infective, particularly that from the nose; infection is normally through a mucous surface and not by an insect carrier. Recovery results in a solid and lasting immunity. Formolised spleen pulp from infected animals confers a high degree of protection. An immune serum can be prepared by immunising cattle with repeated injections of infected blood (hyperimmunity) and this is used therapeutically.¹ Injection of serum together with virulent blood is used to produce immunity in herds. Regarding the virus little is known; it has not been measured or photographed, neither has it been cultivated.

SWINE FEVER

This disease was formerly thought to be due to an organism of the *Salmonella* group—*B. suispestifer*—but a filterable virus is the causal agent. In the acute disease there is a febrile septicaemic infection with haemorrhagic inflammation of the intestinal mucous membrane. In chronic cases there is a necrotic condition of the mucosa in which *Salmonella* organisms are found. The incubation period is about six days.

The natural means of infection is by ingestion, and exceedingly minute doses of virus will cause the disease. The virus is present in the blood plasma and not in the corpuscles.

The virus passes coarse and fine filters, and is fairly resistant. It survives moderate heat, and freezing. It can survive in cured hams and pickled pork, while desiccation does not destroy it. The virus is also resistant to disinfectants and glycerol, but is soon destroyed by putrefaction.

The immunity following an attack is lasting, and hyperimmune serum together with infected blood is used for active immunisation.

ANIMAL POCK DISEASES

Various domestic animals are susceptible to pock-like eruptive skin lesions—namely, horses, sheep, cattle, goats and swine.

Cowpox or vaccinia has already been described (p. 471) and may affect man—e.g. milkers—under natural conditions, while sheep-pox is very closely allied. Inoculation of sheep-pox,

¹ The amount of antibodies in an animal recovered from the natural infection can be increased by repeated injections of infected blood. This method has been termed *hyper-immunisation*, and the serum of the animal is referred to as *hyperimmune serum*.

whether in man, rabbits, cattle, etc., produces immunity to cowpox, and in man also to smallpox.

Sheep-pox is often a very serious disease, and flocks can be immunised with cowpox (vaccinia) or by an admixture of sheep-pox virus and immune serum.

Goat-pox occurs only where the animals are herded. The virus is closely allied to those of sheep-pox and cowpox.

Swine-pox is similar to sheep-pox and cowpox, and may have a mortality rate of 20 per cent. or more.

Horse-pox, known as "grease," affects the mouth, nose and heels. It is uncommon in this country and probably arises from cowpox.

All the animal pox diseases and smallpox are intimately related. There is marked cross-immunity amongst them. The smallpox virus inoculated into calves becomes so modified as to assume the characters of the cowpox virus. Infection with one disease produces immunity to the remainder. It seems probable that cowpox is the original or primordial disease which has affected other animals and man, and become slightly modified according to the animal in which it has become parasitic.

THE ORGANISM OF PLEUROPNEUMONIA OF CATTLE

The specific infective agent of this disease was originally classified with the filterable viruses in view of its ability to pass the coarser filters; it is within the range of microscopic visibility, and can readily be cultivated on an artificial medium. The disease is transmissible experimentally in cattle by subcutaneous inoculation with exudate from the pulmonary lesions and also by the injection of cultures. The organism has been cultivated in serum-broth and on serum-agar.

Ledingham has obtained impression preparations of the colony growths at different stages. These when stained by Giemsa's method show extremely diverse morphological forms, the interpretation of which is difficult. Briefly, the phases of growth are as follows:—

The initial corpuscle extrudes ramifying filaments which tend to coalesce and form more deeply staining nodules or nodes. The nodes in these interlacing mycelial filaments swell up to large oval structures, at first homogeneous, and later dividing into irregular bodies of different sizes which become surrounded by a blue-staining sheath. Later, involution and degenerative changes occur with irregular shapes staining intensely. The definite cycle of growth has not been elucidated, neither has the biological relationship been determined. From the filamentous mycelial growths of the early phases,

Ledingham has suggested that the organism may belong to the *Actinomycetaceae*.

Animals can be immunised against the disease by inoculating infective material or cultures into the point of the tail: injection in this situation produces a non-fatal infection which is followed by immunity for at least a year. The serum of immune animals contains protective antibodies.

AGALACTIA

This infectious disease affects sheep and goats, and is transmitted by contagion. It is characterised by inflammatory lesions of the mammary glands, eyes and joints. It derives its name from the fact that in a lactating animal the milk secretion is replaced by a serous fluid containing clots. The causative organism resembles and is allied to the infective agent of bovine pleuropneumonia. It can be stained by Giemsa's method and is found in arthritic fluid. The organism will pass Berkefeld and the coarsest Chamberland filters. It can be cultivated on serum-agar. Recovery from the infection produces a lasting immunity.

APPENDIX

METHOD OF FILTRATION

It is advisable to filter with as small a pressure as practicable. Suction is the most satisfactory means, the fluid being drawn through the filter.

The following is one of the most convenient forms of *filtering apparatus*. A filter candle (Berkefeld type) is fitted by means of a screw and washers into a cylindrical glass mantle, and the metal tube of the filter passes through the rubber stopper of a conical filter flask of strong glass. This flask is provided with a lateral tube which can be connected with an exhaust pump by pressure tubing. The fluid is poured into the mantle and, after filtration, is collected in the flask. The necessary suction may be obtained by the usual form of water pump, or a Geryk air pump. The negative pressure can be estimated by means of an attached mercury manometer.

By using a low pressure, the time of filtration is prolonged, and this may result in the passage of small motile flexible organisms such as spirochaetes and slender vibrios. When high pressure is used, small particles are rapidly forced into the pores of the candle, thus preventing further filtration. For ordinary purposes, a negative pressure of 100–200 mm. of mercury is sufficient.

When filtering small quantities of fluid, as in dealing with viruses, the Berkefeld candles, size $2\frac{1}{2} \times \frac{1}{2}$ in. (or the small-sized Chamberland filters, sizes 6 or 7, Nos. L_{1a}, L₂ and L₃), are most convenient. In order to secure the maximum amount of filtrate, a test-tube, slightly wider and longer than the candle, should be inverted over it. This ensures that the whole of

the candle is covered with fluid almost to the end of filtration.

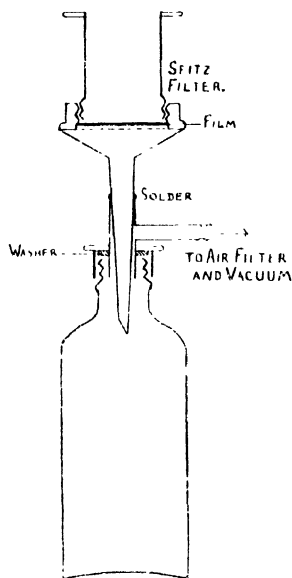
After use, the filters should be brushed with a stiff nail-brush in distilled water, and then boiled in distilled water. Before sterilising again, distilled water should be run through them to show that they are pervious.

Before sterilisation, the glass mantle, the candle and test-tube covering are loosely assembled without tightening up the screw and washers. The open end of the glass cylinder is plugged with cotton-wool, and the metal tube of the candle is inserted into the rubber stopper of the filter flask. The whole is wrapped in kraft brown paper and sterilised by steaming or autoclaving. It is advisable, where several filters and flasks are kept sterilised, to have corresponding numbers on the filters and flasks, to ensure the rubber stopper attached to the filter candle fitting into the correct flask.

The small Chamberland filters are inserted into a rubber stopper so that the open end is flush with the top (*i.e.* large diameter) of the bung. The stopper is now inserted into a small filtering flask so that the candle hangs downward into the flask. Where very small quantities of fluid have to be filtered a small test-tube is placed over the end of the candle before it is inserted in the flask.

In using the Seitz filter the long delivery tube is passed through a rubber bung which fits into a filter flask. The asbestos film should not be screwed down tight. The whole is wrapped in kraft paper and sterilised in the autoclave. It is an advantage to moisten the film with sterile saline solution, and then screw down tightly the upper part of the metal funnel on the softened film before pouring in the liquid to be filtered. It has been our experience that rubber bungs are not resilient after one autoclaving and do not again fit satisfactorily, so that it is necessary to tie down the bung to the filter flask with string and seal the joints with wax. To obviate this the following simple piece of apparatus can be recommended (see diagram). Instead of using filter flasks, screwed white medical "flats," as described on p. 79, are employed (4 oz. for the 3 cm. filter and 10 oz. for the 6 cm. filter). A screw cap is made, into which fits a short piece of thin brass tube (8 mm. bore), having a side arm. The Seitz filter is fitted into the tube and secured with a small amount of solder. To the side arm is attached an air filter, as figured on p. 98. The filter is fitted up, the screw cap is left loose on the bottle, and the whole apparatus is wrapped up in kraft paper and autoclaved. When required for use, the screw cap is tightened up on to the

rubber washer, and the filtration proceeded with as above. Where the filtrate is to be stored—*e.g.* toxin—the ordinary screw cap supplied with the bottle is wrapped in kraft paper and sterilised with the remainder of the apparatus. After



filtration the filter and screw attachment are removed and the ordinary cap removed from its sterile wrapper and screwed on. Where the filtrate is to be kept some time, a viskap (*vide* p. 143) over the screw cap is recommended.

Before filtering nasal washings or other tenacious materials through Berkefeld or Chamberland filters, it is advisable to put them through a sand and paper pulp filter. This can easily be made from a piece of glass tubing 8 in. long $\times$ 1 in. diameter, with the lower end drawn out to a $\frac{1}{4}$ in. bore. At the bottom of the wide portion of the tube is a small perforated porcelain disc, or plug of cotton-wool, and on the top of this is placed about $\frac{1}{2}$ in. of paper pulp which has been well shredded and soaked in water. This is gently pressed down, and on the top is placed about 1 in. of fine washed silver sand. On top of the sand a further $\frac{1}{2}$ in. of paper pulp is placed, and the whole

three layers pressed down. The lower end of the filter fits into a bung and filtering flask. The whole is wrapped in kraft paper and sterilised in the steamer. Filtration is carried out by suction in the usual manner.

This preliminary filtration removes coarse particles, mucus, etc., which would be liable to clog the pores of an earthenware filter.

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