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IMMUNITY PRINCIPLES AND APPLICATION IN MEDICINE AND PUBLIC HEALTH

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IMMUNITY

PRINCIPLES AND APPLICATION IN MEDICINE AND PUBLIC HEALTH

An Exposition of the Biological Phenomena of Infection and Recovery of the Animal Body from Infectious Disease, with Consideration of the Application of the Principles of Immunity to Diagnosis, Treatment, and Prophylaxis and Their Usefulness in the Control of Epidemics

BY

HANS ZINSSER, M.D.
PROFESSOR OF BACTERIOLOGY AND IMMUNOLOGY
HARVARD MEDICAL SCHOOL

JOHN F. ENDERS, PH.D.
ASSISTANT PROFESSOR OF BACTERIOLOGY AND IMMUNOLOGY
HARVARD MEDICAL SCHOOL

AND

LEROY D. FOTHERGILL, M.D.
ASSISTANT PROFESSOR OF BACTERIOLOGY AND IMMUNOLOGY
AND ASSOCIATE IN PEDIATRICS, HARVARD MEDICAL SCHOOL

5th Edition

of

"RESISTANCE TO INFECTIOUS DISEASES"

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PREFACE TO THE FIFTH EDITION

Through the four earlier editions of this book we have successively modified and expanded the presentation of immunology as this was necessitated by the extraordinary velocity of discovery. There was not, however, in preceding revisions any attempt to alter the original plan or purposes of the book, which was conceived as a critical treatise intended chiefly for medical students and laboratory workers.

The present volume, while retaining much of the character of its predecessors, represents an endeavor to meet the need for increased correlation between the principles revealed in laboratories and their applications to the problems of the clinic and of public health.

When the First Edition of this book was written, immunology was still regarded by the medical profession at large as a highly specialized branch of biology with the fundamentals of which the practitioner had little concern, though some of its methods and results had become a part of his professional equipment. This point of view has completely changed in the intervening years. The scientific training of medical students, and consequently of the profession as a whole, has been extended and intensified to such a degree that the barriers which until very recently separated the experimental laboratories from the clinics are gradually disappearing.

The new type of physician insists on understanding the principles of the procedures which are developed by the specialist and of acquiring critical judgment for the evaluation of such methods in his own work.

Out of this new impulse has grown a spirit of co-operation. As never before, the disciplines infiltrate and fertilize each other. The most notable advances of recent years in this subject have resulted from intimate collaboration of the bacteriologist and the chemist on the one hand, of the bacteriologist and the clinician on the other. Medical progress of the future will depend largely on the intensification of such co-operative effort, made possible by improved fundamental education and the consequently increased mutual understanding of principles and objectives by collaborating specialists.

The present completely revised edition is, to some extent, an acknowledgment of this situation. In the section on principles, we have eliminated much of the older material which has become of purely historical value and have added the newer knowledge accumulated

during an extraordinarily fruitful period of eight years. The fundamental nature of many of the discoveries, especially in the chemical definition of antigens and antibodies and the progress in the field of hypersensitiveness, has necessitated complete reorganization of the text. In Section II, on the application of immunological knowledge to medicine and public health, it has been necessary to expand extensively, to re-write most of the chapters completely, and to add several new ones.

If the new volume fulfills its intended purpose, the laboratory worker, turning to the practical sections, may derive stimulus from the recognition that many of the principles at first conceived without obvious possibility of applied value have ultimately served to enrich diagnosis, prevention or therapy; and the practitioner, referring to Section II for details of specific procedure, can turn back to the chapters on principles and there find discussion which will aid him more fully to understand the observations and reasoning upon which many of the methods used in his practice are based.

HANS ZINSSER
JOHN F. ENDERS
LEROY D. FOTHERGILL

PREFACE TO THE FIRST EDITION

Infectious disease, biologically considered, is the reaction which takes place between invading microorganisms and their products on the one hand, and the cells and fluids of the animal's body on the other. The disease is the product of two variable factors, each of them to a certain extent amenable to analysis, and it is self-evident that no true understanding of this branch of medicine is possible without a knowledge of the biological principles which laboratory study has revealed.

For the purpose of helping to render such knowledge easily accessible this book was written. While it is hoped that it may prove useful to the practitioner and laboratory worker, it is intended primarily for the undergraduate medical student. To many it will seem that the subject in general and our method of treatment especially are too technical and difficult for this purpose. Our own experience contradicts this. During the past three years the writer has had the opportunity to deliver lectures and to give laboratory courses on this subject to medical students of 2d-, 3d-, and 4th-year classes at the Stanford and Columbia universities. It has been a pleasant experience to find the medical student eager for the opportunity to obtain this knowledge, and, under the present increased requirements for preliminary training at our best schools, fully capable of assimilating it. It is not a good plan to attempt too extensively to simplify material that, in its close analysis, presents complex phenomena and intricate reasoning. For this reason no attempt has been made to write an A B C of immunity as a quick road to comprehension. No true insight into any branch of medicine or, for that matter, into any other science, can be attained without a certain amount of labor; however, the concepts of this subject are, indeed, relatively simple after the first principles have been mastered, and the writer has attempted, therefore, at the risk of seeming pedantic in places, to treat the subject critically, separating strictly those data which may be accepted as fact from those in which legitimate differences of opinion prevail.

As far as was feasible every chapter has been written as a separate unit. This has necessitated occasional repetition, but, it is hoped, will add considerable to clearness of presentation in each individual subject. Theories have been discussed with as little prejudice as the possession of a personal opinion in many cases has permitted.

The chapter on Colloids was written especially for the book by Prof. Stewart W. Young of Stanford University. Since so many analogies between serum reactions and those taking place between colloidal substances generally have been observed, it has seemed best to devote this chapter entirely to the elucidation of the principles governing colloidal reactions, so that its contents may be utilized as explanatory of the many allusions made to colloids in the rest of the text.

All available sources of information have been freely used. In the large majority of cases we have had access to the original papers and monographs. However, we acknowledge much aid from careful reading of the admirable summaries, written by acknowledged authorities, in the works edited by Kolle and Wassermann, and by Kraus and Levaditi. Similar acknowledgment is made to equally important sources in Weichhardt's Jahresbericht, the Bulletins of the Pasteur Institute, and in such textbooks as those of Paul Theo. Muller, Emery, Adami, Gideon Wells, Marx, Dieudonné, and others. It is needless to acknowledge the use of such classics as that of Metchnikoff or of the many critical writings of Bordet and of Ehrlich — masters who have helped to shape the thoughts of all men working in this field.

The writer takes pleasure in acknowledging many helpful suggestions from his associates, Drs. Hopkins and Ottenberg, and much aid, in the verification of references, from Mr. Walter Bliss, Fellow in the Department of Bacteriology.

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IMMUNITY PRINCIPLES AND APPLICATION IN MEDICINE AND PUBLIC HEALTH

CHAPTER I

INFECTION AND VIRULENCE

THE way to our understanding of infectious disease was first clearly indicated by the studies of Pasteur on fermentation. In fulfillment of the prophecy of Robert Boyle made in the seventeenth century that the problem of infectious disease would be solved by him who elucidated the nature of fermentation, the investigations begun by Cagniard-Latour and by Schwann and carried to a brilliant culmination by Pasteur revealed the living and specific nature of the various microorganisms which cause the several forms of fermentation and especially of putrefaction, and thus led by analogy first to logical speculation, then to experimental proof of the etiological relationship between certain of the minute forms of life and the communicable diseases. The study of putrefaction and of fermentation nevertheless presented a problem far less complex than that of the infection of living tissues with bacteria. For, given any organic material containing suitable nutritive constituents under favorable environmental conditions of moisture and temperature, spontaneously or experimentally inoculated with bacteria or fungi of a suitable species, the phenomena which ensue are essentially those related to the metabolism of the microorganism, in which an active part is played only by the latter, while the organic substrate represents the source whence the chemical compounds and energy essential for growth and reproduction are derived. In the case of infectious disease, however, we have learned that the process is much more involved because here two living entities — the infectious agent and the host — interact with each other. This is made clear from the fact that throughout nature bacteria are abundant, and the environment of man and animals, the outer integuments of skin and hair, the mucous membranes of the conjunctivae, the intestinal respiratory tracts, are constantly inhabited by a thriving bacterial flora. Many of these consist of the ordinarily harmless bacteria (saprophytes), but frequent contacts with many varieties of microorganisms termed "pathogenic" or disease-producing take place without production of manifest disease. Thus perfectly normal individuals may, on occasion, harbor organisms of the latter variety over varying periods of time. The accomplishment of a clinically recognizable infection, therefore, is not determined merely by

the fact that a microorganism finds lodgment in or upon the body of a susceptible individual, but it is further necessary that the invading germ shall be capable of maintaining itself, multiplying and functioning within the living and actively resisting body of the host. We have, thus, a battle of two opposed forces, both intricate in nature, the result of which is infectious disease. It is the initial skirmish between the two which determines whether or not a foothold shall be gained upon the body of the subject, or host, and an infection thus established, and it is the balance between them which decides the eventual outcome of recovery or death. The systematic analysis of these forces in their variable conditions, and of the laws which govern them, constitutes the science of immunity. The facts that have been revealed through immunological researches are as fundamental to the pathology of infectious disease and as essential to the clinical understanding of these maladies as is the knowledge of the mechanism of the circulation, the chemistry of metabolism, or the structural changes of the tissues to the comprehension of other pathological states.

In this and the two subsequent chapters, the principal attributes which have been found to be related to the capacity of the organism itself to cause infection, *i.e.*, its virulence, will be discussed.

Two aspects of the problem of virulence as it relates to the parasite have received by far the greatest degree of study, and these in the last analysis depend upon the chemistry of the bacterial body and its products. The most significant results of these studies which represent immunological fundamentals will be described in the chapters on Antigens and Bacterial Poisons. Nevertheless, variations in virulence occur between strains of a given bacterial species or between the generations of a single strain which cannot as yet always be accounted for satisfactorily on the basis of changes in the chemical or antigenic structure of the parasite or the poisons it may elaborate. Our immediate purpose will therefore be to present a general survey of the problem of virulence and infection and to record what is known concerning these other factors which may be involved in rendering a particular bacterium capable of entering a foreign living body, increasing there, and by its presence calling forth on the part of the host reactions which tend to dislodge or destroy it, and which we recognize as the indications of disease.

The Classification of Organisms According to Their Pathogenic Capacities. One of the fundamental facts, immediately apparent on considering the problems of infection, is the phenomenon that among the innumerable varieties of bacteria and protozoa present in nature there is a very limited group which is capable of becoming parasitic upon the bodies of higher animals, and among these a still smaller proportion which is capable of being "pathogenic" or causing disease.

It is reasonable to suppose that all microorganisms were originally in the condition which we designate by the term "saprophytic." As we have stated, by this term we imply that these germs maintain themselves only upon dead organic matter and do not thrive in or upon the living animal tissues. The class of saprophytes is widely distributed and constitutes, of course, the most important group of bacteria in nature, since upon the activities of these germs depends the unlocking of nitrogen and carbon from the organic complexes in the dead bodies and waste products of animals and plants. Such bacteria if strictly saprophytic, that is, entirely unable to maintain themselves upon living tissues, have on the whole little pathogenicity. Nevertheless, there are cases in which strict saprophytes may cause disease by lodging upon and growing in animal tissues which have been killed by other causes, so-called necrotic areas; and from these through the blood and lymph channels, products of putrefaction or bacterial poisons may be absorbed. While, as a rule, the disease following the invasion of necrotic tissue — such as gangrenous areas, old unhealed sinuses, etc. — may be caused by a large variety of saprophytic bacteria, there are a few very important pathogenic bacteria which are, strictly speaking, saprophytes. Thus the form of meat poisoning caused by the *Bacillus botulinus* is due entirely to the poison formed by this bacillus outside of the body within the substance of the dead foodstuff, and disease ensues as the result of subsequent ingestion of this poison with the food. In the same way the tetanus bacillus and, less strictly speaking, the diphtheria bacillus, at least in its ordinary mode of attack, are closer to the class of saprophytes than to that of the parasites, since neither of these bacteria, under usual circumstances, invades the tissues beyond the point of initial lodgment. The tetanus bacillus, moreover, is not usually capable of maintaining itself and multiplying even at the point of initial lodgment unless the tissues have been injured by trauma, the presence of foreign bodies or pyogenic infection. The condition which ensues is not then, properly speaking, an infection in the sense that invasion of the blood stream by the streptococcus or anthrax bacillus is an infection, but rather a "toxemia," differing from the toxemias resulting from the ingestion of drugs or other poisons only in so far as the toxins are manufactured at some point of bacterial lodgment within the body of the victim. Typical tetanus and diphtheria, for instance, can be produced as readily by injection of the bacteria-free culture filtrates as by inoculation with the bacteria themselves. It should be emphasized, nevertheless, that in the case of many toxicogenic bacteria, including the diphtheria bacillus, the toxin possesses a marked necrotizing action on tissue and as such represents in these species an extremely important means by which a foothold is gained and held within the susceptible host.

With this fact in mind we must consider that such pathogenic saprophytes are endowed with a weapon which permits them to establish an infection, although this does not consist of a massive and generalized invasion of the body by the organism.

In the majority of bacterial diseases, however, which are due to organisms in which the capacity to produce toxic substances is limited, it is necessary that they more or less extensively invade the body before clinically recognizable symptoms are produced. At first sight this group of parasites appear to be distinguished by the different degrees to which in any given species of animal host invasion is effected. Some, such as the anthrax bacillus or the bacilli of the hemorrhagic septicemic group, tend to spread rapidly from the site of entrance to cause a generalized infection of the blood and viscera; others, such as the pneumococcus in man or the staphylococcus, after extending through a certain area of tissue frequently remain localized. With these observations in mind, Bail (1) has classified parasites according to their relative powers of invading the living body. Briefly reviewed, his classification is as follows:

I. *Pure Saprophytes.* (Necroparasites, superficial parasites, or external parasites.)

Microorganisms which under no circumstances can be made to develop within the living tissues of a given animal. This does not exclude their pathogenicity for this animal, since, like the diphtheria or tetanus bacillus, they may develop and produce toxins on a localized area of dead tissues.

II. *Pure Parasites.* Organisms like the anthrax bacillus or the bacilli of the hemorrhagic septicemia group which, implanted in small quantity in an animal, rapidly gain a foothold, thrive, and spread.

III. *Half parasites,* organisms which may be infectious if introduced into the animal body, but, not possessing this invasive power to the same degree as the preceding class, require the inoculation of considerable quantities, often a special mode or path of inoculation, or even possibly a preliminary reduction of the local and general resistance of the infected individual. This class includes the majority of the bacteria pathogenic for man.

Such a classification is, however, quite arbitrary and on the whole of little value except perhaps as a mnemonic device, for even though it be made on the basis of the behavior of the various parasites when brought into contact with a single species of host, no rigid schema is obtained. This is clear from the single example of hemolytic streptococcus infection in man, where the organism may at one time induce a rapidly progressive septicemia with little local reaction, while again it may be confined to a small area such as the naso-pharynx, causing severe angina and even ulceration of the mucous membrane, and whence, in the case of scarlet fever, the absorption of a soluble toxin locally elaborated leads to the distinguishing exanthem.

Because of such considerations it is perhaps best to abandon any attempt at systematic classification of the parasitic bacteria or fungi on the basis of pathogenicity, although a loose division can be made between those organisms which practically always remain localized and exert their effect by the production of a potent toxin, and those which must, in order to cause disease to a greater or less degree, invade the tissues, either through their ability to survive in this living environment or to destroy it through the formation of necrotizing substances or through a combination of both these properties.

Virulence and Invasiveness. In the foregoing paragraphs we have used the terms *virulent* and *invasive* in such a manner that the impression might easily be gained that they were synonymous, and indeed numerous authors have considered them interchangeable expressions, since in many instances those organisms which invade the tissues most widely lead to the most severe conditions and cause the highest mortality. It is, however, evident that were the phenomena of virulence and invasiveness regarded as identical, it would be impossible to refer to a virulent diphtheria bacillus or a virulent tetanus bacillus, neither of which, as we have seen, ordinarily invades the tissues to any great extent. However, avirulent strains of both are not infrequently encountered. Menkin (2) has recently emphasized the importance of distinguishing clearly between the capacity of an organism to invade and its virulence, and has presented experimental data which show that these two attributes of a given bacterium (Pneumococcus Type III) can be distinguished. We are inclined to regard the term *virulence* as being equivalent to *pathogenic*. Invasiveness, then, would be left to signify that property of certain organisms which permits them to spread through the tissues and body fluids of the host. Accordingly, in certain cases virulence or pathogenicity will be found to be conditioned principally by the characteristic of invasiveness, while in others toxigenicity will be the determining factor; in still others the organism may depend for its pathogenic effect on the possession of both characteristics.

Relationship between Virulence and Dosage. It is self-evident that there must be an inverse relationship between the virulence of microorganisms and the numbers or dosage which can bring about infection. For, even when the bacteria are of a variety known to produce disease, and are brought into contact with the body by a path suitable to their peculiar requirements, the initial quantity introduced must be sufficiently large to preclude immediate annihilation by the defensive powers of the body, which are usually but not inevitably present to a greater or less degree. It is plain, therefore, that in the case of bacteria weak in power to cause disease, given the subject of infection and his defenses as a constant, the quantities to be introduced must be larger than in the case of microorganisms of violent disease-producing properties. Thus we measure the degree of the so-called virulence of bacteria by determining the smallest number which will still cause infection and death in susceptible animals of a standard

weight and strain. In the case of microorganisms of extreme virulence, such as the anthrax bacillus or bacilli of the hemorrhagic septicemia group, the inoculation of a very small number of bacteria may suffice to initiate infection. Indeed, the injection of a single anthrax bacillus of sufficient virulence may produce fatal disease in a susceptible animal such as the mouse. The experiments of Goodner (3, 4) in our laboratory have shown a similar thing with pneumococcus infections in rabbits. Type I Pneumococcus will cause death in rabbits by the intracutaneous route even when only a single organism is introduced.

Contrasting with the maximum virulence of such bacteria is the relatively low pathogenicity of freshly isolated strains of meningococci or typhoid bacilli for mice. The smallest dose of the former which will kill when injected into these animals must be measured in milligrams of bacterial growth (5), and even with the moderately virulent typhoid bacillus, Grinnell (6) found the lethal dose for 80 per cent of mice tested to contain about 30,000 individual bacteria. In these cases where large doses are required, although the organisms have been obtained directly from human cases, and in all probability are equipped with the full complement of pathogenic properties, the factors involved in the natural resistance of the host which resist the attack of the bacteria without much doubt account for the low degree of virulence which they exhibit. Frequently, however, a given species of organism may itself become altered in some way whereby the virulence as measured by the number of bacteria in the minimum lethal dose is greatly decreased. This can occur through the loss of the capacity to synthesize certain protective (to the bacterium) antigenic chemical constituents — a phenomenon which will be subsequently described. In certain instances, however, the virulence may rise or fall independently of any apparent change in antigenic structure. Eaton (7), for example, has described certain strains of Pneumococcus Types I, II, and III which became markedly reduced in virulence for mice, but remained in all other respects identical with the fully pathogenic organism. Practically avirulent pneumococci which retain all their type specificity in respect to agglutination have likewise been studied by Schliemann (8). Wilson (9) has observed a similar phenomenon with *Bacterium Aertrycke*. Obviously the dosage of such "attenuated" bacteria required to bring about the death of a mouse will be large.

That fluctuations in virulence associated with some change in the organism may occur very rapidly which are at once reflected by the increase in the minimum lethal dose is well illustrated by the careful experiments of Webb, Williams, and Barber (10) carried out upon white mice with anthrax bacilli, using the single cell isolation method devised by Barber.

This technique consists in picking up single organisms with a capillary pipette under microscopic control, from a very thin emulsion of bacteria and injecting directly from the pipette through a needle puncture. While requiring a considerable degree of skill, the method permits an accurate count of injected bacteria instead of the approximate estimate of consecutive dilutions. They found that the inoculation of a single thread of anthrax bacilli (3 to 6 individuals) taken directly from the blood of a dead animal would regularly cause death, and it was impossible for this reason to immunize with such bacilli. On the other hand, if taken from 12-hour agar cultures of the same strain such quantities would often fail to kill. The brief period of growth under artificial conditions had sufficiently lessened the virulence of the bacilli so that 2, 3, and more threads could be injected without harm. And after several generations of such cultivation as many as 27 and more threads could be inoculated with impunity.

It would seem that under natural conditions the spread of many infectious diseases is conditioned to a considerable degree by the dose of the infecting agent, although, of course, other variables of which we are only beginning to have any precise conception play important parts.* But the difficulties in obtaining accurate data from which definite conclusions can be drawn concerning the relation of virulence and infectivity to dosage in these cases of spontaneous infection are readily apparent. We can only surmise that with highly contagious diseases such as measles the inoculum required to produce the condition is small, whereas with others, less readily contracted, such as whooping cough, greater quantities of the pathogenic agent must be brought in contact with the susceptible individual; although here again differences in species and individual resistance may represent the decisive factor. The comparatively small body of experimental evidence that we possess would indicate that the quantity of available organisms is a determinative factor in the epidemic process. In populations of white mice infected with *B. enteriditis* Webster (11) has found that the epidemic mortality varied directly with the available dosage at a time difference approximating that of the incubation period of the disease. The dosage was measured by determination of carrier rate and the actual number of *B. enteriditis* in the bedding on various occasions. This author has recorded similar correlations between dosage and epidemic severity in experimental epidemics caused by Pasteurella and a Friedlander-like organism.

Changes in Virulence Induced by Alterations in the Environment. Adaptation to the Environment of the Host. Fluctuations in virulence of a given strain of a parasitic species have long been known to occur readily. Decrease in pathogenicity is indeed almost the rule with bacteria kept on artificial media in the laboratory with no precautions

* For a discussion of the relationship between dosage and epidemic spread see the papers by Neufeld (12).

to maintain the virulence. Evidently, as the organism accustoms itself to such conditions, it tends to become less well adapted to the peculiar conditions under which it was formerly able to thrive in the body. Conversely, complete restoration of pathogenic power can often be brought about by repeated passage through an appropriate animal. This procedure frequently results in also increasing the virulence of an organism for a species of host to which it is not naturally adapted. Thus with streptococci and pneumococci, organisms in which no two strains need be alike in infectiousness, and in which the injection of certain races into susceptible animals may produce no effect whatever, the pathogenicity may be tremendously enhanced. In the case of streptococci, strains which may not kill mice in quantities of 1.0 cc. or more of an eighteen-hour broth culture, when first isolated from an infected human being can, by many passages from mouse to mouse, become so virulent that 0.00001 cc. of culture will kill. It is impossible to accomplish this with every strain of this species, and of a dozen strains one may succeed only with two or three.

Among the earliest observations on this phenomenon are those of Pasteur (13) in his work on rabies. He found that the virus of hydrophobia when successively passed through rabbits gained in virulence until a degree of maximum infectiousness was attained beyond which it could no longer be enhanced. After only three passages through monkeys, however, the virulence of this "virus fixé" for rabbits was reduced almost to extinction. Yet the same virus, passed through dogs, as in "street virus," remains virulent for rabbits. His experience with swine plague was similar. Swine plague bacilli successively passed through rabbits and pigeons gained in virulence for these animals, but lost in virulence for hogs.

There are numerous other methods by which the virulence of microorganisms can be attenuated. Most of these consist in subjecting the bacteria, in artificial culture, to deleterious influences, unfavorably high temperature, exposure to light or harmful chemical agents, or allowing them to remain in prolonged contact with the products of metabolism by infrequent transplantation. As a rule the attenuation which follows any form of artificial cultivation in the case of bacteria like streptococci or pneumococci can be delayed by preserving them in media containing blood. Based on the fact, long since recognized, that drying in the cold tended to prolong viability and virulence, during recent years various techniques have been developed which are of great value, not only in preserving bacteria and viruses in their original state for long periods of time, but also in enabling us to prevent other labile materials such as antisera from undergoing rapid deterioration. The details of such procedures, as well as those employed in attenuating organisms for use in the preparation of vaccines, will be described in their appropriate place under the discussion of the immunity to the various individual parasites.

It is interesting to consider adaptation of the organism to the environment of the host such as we have described above as a sort of immunization of the bacteria against the defensive powers of the latter — a conception early suggested by Welch. For just as the animal body may become more resistant to the offensive weapons of the invaders, so, it is reasonable to suppose, the bacterial body may develop increased resistance to the defensive mechanism of the host. And this, if it occurs, would lead to an increase of invasive power and virulence. The increase of virulence by passage through animals would alone lead us to suspect that such acquired resistance to destructive agents on the part of the bacteria might be responsible for the enhancement. Experiments intended to simulate this process *in vitro* were carried out by Walker (14). Walker cultivated typhoid bacilli for a number of generations upon the serum of a typhoid-immune animal, and found that after such treatment the organism had gained in virulence and lost in agglutinability by immune serum, and that a larger amount of specific immune serum was necessary to protect animals against it than sufficed for protection against normal typhoid strains. Similar observations (15, 16, 17, 18) can be found recorded in the older literature. In the light, however, of our newer knowledge concerning the phenomenon of bacterial dissociation or variation, we recognize that one of the most reliable means of inducing changes in the direction of a lessened virulence is to grow the organism in its specific antiserum. It may be that there are exceptions to this method of attenuation, but this seems unlikely, for, as Hadley (19) asserts, the conflicting results can probably be explained by variation in the concentration of antiserum employed to effect the change.

Importance of the Path of Entrance. A phase of parasitic adaptation of considerable importance is the dependence of successful infection upon the path of entrance. Even when an organism belongs to a species pathogenic for the given animal, is virulent, and enters in adequate amounts, it may not infect unless it enters the host by the particular path to which it has become pathogenically adapted. Organisms entering by the intestinal canal are subjected to conditions of acidity or alkalinity, the action of digestive juices, of bile, and to competition with other intestinal bacteria, forces to which many pathogenic germs will succumb, while others may survive there and thrive. Those entering the tissues by way of the skin and mucous membrane, on the other hand, encounter an immediately mobilized protective mechanism which, successfully resisted by some of them, may easily and quickly dispose of small quantities of other bacteria more resistant to conditions in the bowel.

The mechanical protection afforded by the coverings of skin and mucous membranes is as a rule sufficient to prevent the penetration of any bacteria which by chance find lodgment upon them. In the case of the pyogenic

cocci and many bacilli such protection is probably absolute, and a break of continuity, a bruise or a wound, even though this may be too small to attract attention, is necessary for successful infection. In the case of a very limited number of diseases infection seems to take place through the unbroken skin, and the method, often spoken of as the vaccination method of Kolle, employed to produce experimental plague in rats or guinea pigs, consists in merely rubbing a small amount of cultural material into a shaven area of the skin. However, in this case, as well as in other instances where mere massage of bacteria into unbroken skin has led to successful inoculation, it is more than likely that success has depended upon either microscopic lesions or introduction of the organisms into the sebaceous glands, the sweat glands, or hair follicles. The defense of intact mucous membranes, however, is by no means impervious. While many organisms can be implanted upon mucous membranes with impunity, there are a number of others that can cause local inflammations upon these and pass through them. Thus gonorrhea is ordinarily a disease of implantation upon a mucous membrane, and diphtheria bacilli and streptococci give rise to localized disease on the pharyngeal and nasal mucosae, the latter not infrequently penetrating from the initial point of lodgment into the deeper tissues and the circulation, causing "septicemia" or "bacteriemia." It has been shown that plague (20) and glanders (21), as well as hydrophobia, may be transmitted by simple instillation of infectious material into the uninjured conjunctival sac. In the case of hydrophobia (22) a young man contracted hydrophobia by rubbing his eyes with a finger contaminated with the saliva of a rabid dog. In syphilis, there is no positive proof to show that infection may take place through the uninjured surfaces. Even without the existence of demonstrable morphological lesions, the penetration of microorganisms may be made possible by functional injury, such as congestion or catarrhal inflammation.

However, it is only very definite species of microorganisms which can cause disease at all when introduced into the body by these paths. For, although the rubbing of plague bacilli into the skin, or the inoculation of a cut surface with streptococci or glanders bacilli, will lead to progressive infection, similar inoculation with the typhoid bacillus or the cholera spirillum would have no such result. And, though the swallowing of pyogenic cocci, pneumococci, and a number of other microorganisms would be entirely without effect, similar ingestion of the typhoid and cholera organisms usually result in typical infection. In the case of cholera the spirillum is peculiarly susceptible to the deeper defenses residing in the body fluids and cells, and cutaneous infection is promptly checked. In the intestinal mucosa, however, the cholera spirillum finds conditions favorable for rapid multiplication and the disease is caused by the inflammation and destruction of the mucous and submucous tissues by the poisonous substances emanating from the spirilla which die and are disintegrated, as well as by the absorption of these poisons into the circulation. The bacteria themselves, however, usually do not gain a permanent foothold within the blood or other

organs, although this may occur in some instances. In the case of typhoid fever the conditions are somewhat similar, although here, during the earlier weeks of the disease, we have with great regularity a penetration of the bacilli into the circulation.

More recently recognized and most interesting examples of this relationship between virulence and mode of entrance are those afforded by the findings of Shope (23) on the virus of swine influenza, those of Andrewes (24) and his coworkers and of Francis (25) on that of human influenza, and of Webster (26) on rabies. The active agent of swine influenza fails to cause clinically apparent disease when inoculated other than by the intranasal route, although it produces a definite effect since an immunity can be demonstrated following its subcutaneous introduction into the body. Similarly with the virus of human influenza frank disease in ferrets and mice occurs only after intranasal inoculation. The virus of rabies grown in tissue culture and then injected intracerebrally into mice brings about illness and death, but when placed in the peritoneal cavity it is without obvious effect. But here again, immunization of the animal results from contact with the virus.

Not only do the various species of infectious agents reveal differences in adaptation to a given pathway of entrance into the host, but even individual strains of the same organism may show a predilection for one route over another, as is indicated by the work of Webster and Clow (27). These authors found that a strain of pneumococcus of high virulence for mice when injected intraperitoneally might be of low killing power when introduced intranasally. Moreover, they observed that strains of initially high intraperitoneal and intranasal virulence when repeatedly passed in mice by the nasal route proved avirulent when intranasally injected, but retained their original intraperitoneal virulence.

The analogous behavior of a filterable virus is well illustrated by the observations of Levaditi and his colleagues (28) on vaccinia. Most strains of this virus are "dermotropic," *i.e.*, they appear to have a marked tendency to localize in the skin. Rabbits are only slightly susceptible to strains of this kind inoculated intracerebrally. Levaditi found, however, that an original dermotropic virus could be made neurotropic by repeated passage through the brains of rabbits. The newly acquired properties conferred on the virus the capacity regularly to cause encephalitis resulting in the death of the animal. Inoculated by a route other than the cerebral, the virus could be recovered from the brain in contradistinction to the findings with dermotropic strains which could not be demonstrated in nervous tissue.

These few examples, selected from a large body of experimental and observational data which all testify to the same fact, should make it apparent that the capacity of an organism to meet and overcome the conditions

encountered on its first contact with the host is an important factor in the determination of pathogenicity. That which is still largely obscure, however, is the nature of these local host conditions which are opposed to the invader. In addition to the mechanical barrier presented by the skin and mucous membranes, other mechanisms of a defensive nature associated with these tissues have been described. Such recent observations as those of Arnold and Gustafson and others (29) concerning the bactericidal properties of the normal human skin indicate that certain varieties of bacteria (hemolytic streptococci, *Ch. prodigiosum*, *Bact. typhosum*, *Bact. coli*, *Bact. enteriditis*) were rapidly killed (within ten minutes in some cases), when placed, for instance, on the clean skin of the hand. If much dirt is present, this bactericidal mechanism is far less efficient. In contrast to the rapid disappearance of these bacteria which are not normal inhabitants of the dermis, it was found that such species as staphylococcus or certain of the diphtheroids which are constantly associated with the skin, underwent no decrease in numbers. The exact mechanism by which the pathogens were destroyed has not been determined. It is possible, as Arnold believes, that the low *p*H of the skin (5.8–5.2) represents an important factor. It may well be, as Topley (30) suggests, that some constituent of the sweat may account for this autosterilizing effect. Stevens (30 a) has lately studied the bactericidal properties of ultraviolet irradiated lipids of the skin, and finds these substances after exposure to ultraviolet light capable of killing such organisms as the hemolytic streptococcus. This effect is most probably due to the release of active oxygen on contact with the bacteria.

As has been remarked above, in the digestive tract the various secretions such as saliva, mucus, gastric juice actively function in preventing certain parasites from penetrating the epithelial lining and gaining access to the tissues. This effect may in part be accomplished by the purely mechanical act of washing. Thus as Bloomfield (31, 32, 33, 34) has demonstrated, carbon particles and bacteria placed on the tongue, sublingual spaces, or tonsils are rapidly removed, most probably by the flushing action of the saliva, in which currents are set up by the act of swallowing. As with the skin, bacteria which are not members of the normal flora of the tongue swiftly disappear, while no measures that were tried resulted in the removal of the organisms indigenous to this site. There can be little doubt that in these experiments the organisms are swallowed with the saliva.

Having reached the stomach, the organisms are at once subjected to a strongly acid environment which doubtless kills many species (35, 36). The bactericidal action of gastric juice was apparent to the early investigators. Thus Koch (37), in attempting to produce disease in guinea pigs by the administration of cholera *vibrios per os*, sought to overcome its effect on these organisms by giving large doses of sodium carbonate before inoculation. The autosterilizing properties of the intestinal tract have been extensively studied by Arnold and his associates (38, 39, 40). In a series of papers they have given more precise data concerning the manner in which this entirely non-specific immunological mechanism acts. By suturing various portions of the stomach and intestines to the abdominal wall in dogs, samples of the gastro-intestinal contents at these various levels could be removed from time to time, tested for *p*H, and bacteriologically examined in respect to the normal flora and the presence or absence of other bacteria introduced *per os*. Correlated with a rising *p*H in passing

from duodenum to the ileum was an increase in numbers of bacteria representing normal inhabitants. Differences were also observed in the distribution of various species. In the duodenum and upper half of the jejunum where the *p*H of the intestinal tract was lowest (5.2–6.5) only a few Gram-positive cocci were found. In the lower half of the jejunum (*p*H 6.0–7.0), in addition to Gram-positive cocci, Gram-positive and Gram-negative bacilli were observed. In the ileum (*p*H 6.8–8.0) a very numerous and varied bacterial population was encountered. When neutral suspensions of such species as *Ch. prodigiosum* and *P. pyocyoneus* were introduced into the stomach of a fasting dog, they could not be recovered in the content of the caecum. However, if they were given in an alkaline buffer solution, they readily arrived at this site.

Just as in the mouth and pharynx the saliva plays an important role in the removal of small particles, so in the gastro-intestinal tract the mucus takes up bacteria and, propelled by the peristaltic movements of the bowel, carries them onward. Florey (41) has investigated and described the details of this process. From his observations it is clear that with non-pathogenic bacteria the mucus represents an important means in their disposal. With a virulent organism in a susceptible host such as *B. Aertrycke* it fails to protect the mucosa in certain areas as is evident by the appearance of inflammatory reactions characterized by leucocytic exudation and the focal destruction of epithelial cells.

Bacteria settling on the conjunctivae are in large measure removed by the washing effect of the tears (42, 43). That they may in part be destroyed by the so-called lysozyme of Fleming (44, 45) is a likely possibility. This principle found by this author in tears, nasal secretions, tissue extracts, and egg white has the power of lyzing many non-pathogenic bacteria and a few strains of staphylococci and streptococci of human origin. While particularly effective in destroying an organism termed by Fleming "Micrococcus Leisodeikticus," it is probable that this substance has little or no importance in overcoming virulent species of organisms, as it has practically no effect upon them *in vitro*.

The anatomical structure of the anterior nares tends to obstruct the passage of many inhaled bacteria (46), while those that penetrate more deeply come to lodge in the mucus of the mucous membrane, which, propelled backwards by the ciliated epithelium of the nasal mucosa, joins the material coming from the mouth and is swallowed. Lysozyme present in high concentration in nasal secretion may also assist in the removal of saprophytic bacteria. That some sort of bactericidal mechanism appears to be present is strongly suggested by the rapid disappearance of bacteria sprayed into the nose noted by Arnold, Ostrom, and Singer (47) which they could not account for on the basis of mechanical flushing.

Under the conditions of normal life, it is likely that the number of organisms which gain access to the deeper portions of the respiratory tract is limited. Their progress down the trachea and bronchi appears to be inhibited by the retrograde action of the living film of mucus set in motion by the ciliated epithelium. There is a certain amount of experimental evidence which suggests that pathogenic organisms may reach the lung parenchyma, tissues, blood, and lymph by penetration through the mucosa of the upper respiratory tract (48, 49).

It is of interest to note that the naso-pharyngeal mucosa may be arti-

ficially altered by the application of such substances as tannic acid and sodium alum in such a manner that the capacity of certain viruses to pass this barrier is definitely decreased or inhibited (50, 51, 52, 53). The exact nature of this alteration is unknown and the increase in resistance to this virus does not extend to other parasites such as bacteria like the pneumococcus, as is demonstrated by the experiments of Rake (49).

The sterility of the mucous surfaces of the normal urethra except near the external orifice is probably largely maintained by the flushing action of the urine as well as its somewhat acid reaction (*p*H 6.0). The latter factor seems to assist in preventing the growth of many sorts of bacteria on the vaginal surfaces, which, however, maintain a characteristic flora of aciduric bacteria of the Döderlein type.

In many cases a pathway of entrance is afforded by certain accidental factors which, by providing suitable conditions, make it possible for the organism to enter the body and proceed to increase in numbers. Trauma is the most important of these. It is well known that injury in which tissue is killed favors the development of streptococci and staphylococci. In tetanus and other anaerobic infections, this is an essential factor (54), since upon it depends the reduction in oxygen tension requisite for the proliferation of the organisms (55, 56).

In addition to trauma the presence of concomitant or pre-existing infection by another species of pathogen frequently prepares the way for secondary invaders of tissues which if entirely normal would be capable of repelling their attack. This preparation, just as with trauma, may take the form of tissue destruction and the production of an area of reduced oxygen tension such as is caused by the staphylococcus and which affords an excellent milieu for the development of the tetanus bacillus as has been shown by Francis (57), among others. Entirely unknown is the nature of the environmental changes due to such epidemic diseases as influenza, measles, and whooping cough, which appear to favor subsequent severe and fatal infections with pneumococci, streptococci, and influenza bacilli. It is true also of many varieties of encephalitis that the penetration of a virus into the central nervous system is almost always secondary to a preceding debilitating condition — influenza, measles, mumps, smallpox, etc.

Further examples of what might be termed symbiotic enhancement of virulence are the association of diphtheria bacilli and streptococci in the throat and of the organisms of Vincent's angina with diphtheria. In both these cases the clinical diphtheria is apt to be considerably more severe than when uncomplicated by the presence of other than the ordinary bacterial inhabitants of the nose and throat. Still another instance of association in which one agent increases the pathogenic power of a second is afforded by swine influenza, in which a filterable virus capable when injected alone of causing only a mild disease, is able to produce a severe pneumonia with marked systemic symptoms expressed by high fever and prostration when accompanied by the bacillus of swine influenza (58). Of probably similar significance, also, are the conditions prevailing in hog cholera where the so-called hog cholera bacillus is almost always present, although we know that the specific etiological agent is quite a different organism — a filterable virus.

Numerous instances of the stimulatory or inhibitory effect of one species of bacteria upon another when growing in culture are recorded in the litera-

ture. Even under these relatively simple conditions the factors which account for the observed phenomena are difficult to discern. As Holman (59) points out, changes in *p*H, production of hydrogen peroxide catalase, carbon dioxide, and various amino acids, may well be involved. One might offer the conjecture that similar factors may be operative in the body infected with two organisms, but there is practically no direct evidence to support it.*

Virulence and Capsule Formation. A characteristic of certain species and strains of bacteria which may well be regarded in some cases as a manifestation of adaptation to parasitic existence, as probably was first suggested by Babes (60), consists in the formation of a capsule. This structure in such organisms as the various types of pneumococci and Friedländer bacilli appears to be composed of various kinds of complex carbohydrate gums. That produced by the anthrax bacilli is described by Tomcsik (61) as a protein or protein-like substance. It extends about the somatic portion of the bacterium and can be sharply differentiated from this vegetative body by suitable staining procedures. It is also visible when the living organism is viewed in the dark field or mixed with india ink.

The experiments of Danysz (16) were among the first to indicate a definite relationship between the formation of a capsule and virulence in pathogenic bacteria, and many subsequent studies have in the main served to establish this structure as being of great significance in permitting the survival of the bacterium *in vivo*. Its presence, nevertheless, cannot be taken as an infallible sign of an organism's capacity to cause disease, since a number of harmless encapsulated saprophytes are known. Yet in the realm of the pathogens it is a matter of common observation that microorganisms like the pneumococcus, the anthrax bacillus, some streptococci, and a number of others which are capable of producing capsules under certain conditions are most virulent when the capsule is fully developed. If the ability to encapsulate is lost, as it frequently is when dissociation occurs, or after prolonged growth on artificial medium, the virulence becomes markedly reduced or entirely disappears. When strains of lowered virulence are passed through animals, their pathogenicity increases *pari passu* with the development of capsules. Organisms like the Friedländer bacillus, which retain their ability to form capsules for prolonged periods of time on artificial media, do not lose their virulence as long as this function is preserved. In addition to this direct evidence for a close correlation between virulence and capsule formation, there exist a number of observations obtained by test-tube experimentation which lend it further support. Early in the study of the significance of encapsulation such workers as

* For further details concerning bacterial association consult the review by Holman (59).

Porges (62), Shibayama (63), and Fitzgerald (64) noted the failure of capsulated bacteria to be clumped or agglutinated (see chapter on Agglutination and Precipitation) by an antiserum capable of inducing this phenomenon to a marked degree in non-capsulated organisms of the same species. Since such clumping is brought about by an antibody in the serum which appears in response to the presence of the given organism in the body of an animal the function of which appears to be directed toward the destruction or removal of the parasite, the failure of an organism to be agglutinated suggests that the capsule represents an important factor in resisting this particular mode of attack by a property of the host's blood serum. Such a conception is further supported by the demonstration of V. Eisler and Porges (65), in which they removed the capsules of inagglutinable bacteria by treatment with acid and thus restored their susceptibility to clumping by an appropriate antiserum.

Not only do certain sera contain agglutinating substances, but they also possess the property under definite conditions, which will be subsequently analyzed, of dissolving, lysing, or killing microorganisms of various sorts when mixed with them *in vitro*. It was Danysz (16) again who first noted that encapsulated bacteria remained unharmed in contact with sera actively lytic against non-capsulated forms. His observations were supported by the findings of earlier authors, and of later years by such as those of Ward and Wright (66), who studied the lytic effect of normal human serum on virulent and avirulent influenza bacilli. Since virulent strains of these organisms have been shown by Pittman (67) to be equipped with a capsular envelope, it is reasonable to believe that their resistance to the lytic action of the serum may in part be conditioned by the possession of this structure or its components. The relationship between the antiserum reactions and capsule will become clearer after the reader has familiarized himself with the fundamental knowledge concerning the nature of antigens.

Encapsulation also renders bacteria insusceptible to that property of serum which prepares various foreign particles and microorganisms for ingestion by the phagocytic cells of the body. (For a detailed account of the various types of phagocytic cells see the chapter on Phagocytosis.) Grüber and Futaki (68) recorded the failure of phagocytes to take up anthrax bacilli after they had developed capsules in the animal body, although phagocytosis of the same organisms readily occurred when they were introduced in a non-capsulated state, such as is found in the ordinary broth culture. Bordet (69), Hess (70), Wreschner (71), and Ward and Lyons (71 a) have reported similar differences between capsulated and non-capsulated organisms. Ward and Enders (72), studying the phagocytosis of young and old cultures of Pneumococcus

Types I, II, and III by normal human sera and leucocytes, found that six- to eight-hour cultures which exhibited large numbers of encapsulated cocci were less readily ingested than those which had been incubated for eighteen hours or longer. In the extremely susceptible rabbit, Enders, Shaffer, and Wu (73) noted that the completely encapsulated organisms from young cultures of Pneumococcus Type III following injection remained in the circulating blood in contrast to the rapid removal of those which had been allowed to continue their growth *in vitro* for a more prolonged period. Since the elimination of the latter was found to be effected mainly by the phagocytic action of the fixed tissue cells, it was concluded that the possession of the capsule in an unimpaired condition was the essential factor in permitting the organism to remain and increase in the blood stream.* These studies, as well as the much earlier ones of Preisz (74), also emphasize the fact that not merely the power to produce capsules, but also the faculty of maintaining them intact over a relatively prolonged period of time, may be correlated with the virulence of a strain for a particular species of animal. It would appear as if the metabolic processes of various pathogenic strains of the same species upon which the elaboration and maintenance of the capsule must depend, revealed marked differences which are manifested in varying degrees of pathogenicity.

Not only does the capsule protect the organism in the ways which we have seen against the biological defenses of the host, but it also seems to increase its resistance against the noxious effect of certain chemical agents such as arsenic (16) and phenol (76).

As yet capsule formation has not been definitely demonstrated in all species of pathogens. Future investigation may reveal its presence in some of these, since in the recent past capsules have been observed in such bacteria as *H. influenzae* (67), *H. pertussis* (77, 78), and many streptococci (79) which were formerly regarded by many workers as non-capsulated varieties. Until 1914 and Rowland's (80) description of encapsulated plague bacilli there was some doubt as to whether these organisms were capable of exhibiting this characteristic.† In 1931 Gilbert (81) described a capsulated staphylococcus. Encapsulated typhoid bacilli have been observed by a number of workers (82), and it is not difficult to conjecture that the inagglutinability of the so-called "Vi" or virulent strains of this organism in certain antisera recorded by Felix (83) in 1935 may ultimately be found to be associated with

* In this connection should be cited the experiments of Felty and Bloomfield (75), who showed that young cultures of pneumococci were more virulent for mice than those which had been incubated for a longer period.

† The report of the German Plague commission (20) published in 1899 describes capsules on plague bacilli taken from the animal body and gives excellent photographs showing these structures.

the capacity to form a capsule of a particular chemical nature. Churchman (84) by means of a special staining technique has demonstrated appearances resembling capsules in a large variety of bacteria not ordinarily credited with their possession. Although one might be inclined to regard some of these as artifacts, many seem to reflect a definite morphological attribute. Even though ultimately a class of organisms may still remain distinguished by the absence of a capsule, it is nevertheless possible that in these cases changes in the peripheral portion of the bacterial cell may take place analogous to true encapsulation. This conception has been upheld by Eisenberg (85), who based his thesis on the fact that non-capsulated bacteria at times show swelling or enlargement under conditions in which their offensive activities are called into play. Such forms Bail has spoken of as "thierische Bazillen," because this appearance is most frequently encountered after residence in the animal body. Eisenberg looked upon the hypertrophic outer zone of these organisms as a protective layer.

From what has been stated concerning the capsule of pathogenic organisms, it is apparent that under certain conditions it does act as a protective envelope which interposes a barrier between the somatic portion of the microbe and the forces which tend to destroy it. Its defensive function, however, must be regarded as limited, for the resistance of capsulated bacteria to the action of a given serum would appear to depend upon the absence in the serum of an antibody which will react with the capsular constituents. Provided that such antibody be present in sufficient quantity, agglutination, lysis, or phagocytosis will generally ensue. This may be illustrated by the fact that in the presence of normal serum and cells of the susceptible rabbit or in that of infants under one year of age, phagocytosis of capsulated pneumococci is absent or occurs to a minimum degree, although non-capsulated forms are easily taken up by the leucocytes. When the rabbit is immunized by injecting virulent killed pneumococci containing the capsular substance, or when the child with increasing age naturally develops antibody against this material, the ingestion of capsulated cocci readily takes place when brought into contact with the cells and serum from either the artificially immunized animal or the naturally immunized human being.

Virulence and Adaptation to Body Temperatures. The conception that the high normal body temperature of certain species of animals presents an unfavorable environmental condition for the survival or multiplication of certain organisms pathogenic for other hosts with lower temperatures, has been in the minds of investigators and clinicians since the experiments of Pasteur demonstrated that the chicken, normally refractory to anthrax, became susceptible after the temperature had been lowered by immersion in cold water. The immunity of birds to the human and bovine varieties

of the tubercle bacillus has often been attributed to their high normal body temperature (about 104° F.), since these organisms grow poorly and cease to multiply when subjected to temperatures of 107° and 108°.* The low temperatures of cold-blooded animals appear to represent a condition favorable to the development of cold-blooded strains of tubercle bacilli which have a growth optimum at about 25° C., and which fail to cause disease in animals or birds. References to the older literature on a possible relationship between virulence, temperature, and infection are given by Seitz (86).

Due to the stimulus afforded by the report of Wagner-Jauregg (87) in 1918 concerning the use of malaria in the treatment of dementia paralytica, a considerable number of more recent studies have appeared on the effect of temperature on the growth and viability, both *in vitro* and *in vivo*, of various organisms. These newer data illustrate more accurately and completely the delicate adjustment of certain species and strains of microorganisms to this environmental factor. The work of Carpenter and his colleagues (88) has, for example, defined the conditions of time and temperature which lead to the destruction of the gonococcus *in vitro*. Clinical application of these findings in the treatment of gonorrheal urethritis and its complications by artificially raising the body temperature to 41° to 41.7° C. has yielded results which, in this case at least, seem to be correlated with the experimental observations, since such therapy is often attended with the disappearance of gonococci from urethral discharges after the temperature of the patient has been maintained for a few hours at about 41° C. (89). Carpenter, moreover, found considerable variation between different strains of gonococci in their capacity to withstand increases in temperature above 37° C. Desjardins (90) notes that in certain instances the superior resistance of some strains appeared to account for the failure of the patient to respond to fever therapy. Analogous observations on the degree to which adaptation to temperature has been achieved among various strains of the same organism have been recorded by Enders and Shaffer (91), who found that strains of Pneumococcus Type III obtained from cases of pneumonia and meningitis might be distinguished by their ability to survive and multiply at 41° C. Most of those which resisted this temperature were found to be virulent for rabbits, while those which succumbed failed to produce fatal disease when injected into these animals. Rich and McKee (92), working with the same organism, reported similar findings in rabbits. Interesting examples of apparent adaptation of a single strain of an organism to higher temperatures with a concomitant increase in virulence are presented by the experiments of Bessemans, Van Haelst, and others (93) on *Treponema pallidum* and Armstrong on vaccinia virus (94). The former authors showed that the treponema localized in the inguinal and popliteal lymph glands of rabbits and guinea pigs inoculated with syphilitic material, and in the spleen and brains of infected mice, were more resistant to heat than those occurring in dermal lesions, and were characterized by the highest degree of pathogenicity, since one organism was shown to be capable of initiating the experimental disease. According to

* Duncan and Mariette (85 a) have recently redetermined the temperature at which the various varieties of tubercle bacilli cease to grow. Their mammalian strains failed to multiply above 102° F., while the avian variety continued to increase at temperatures up to 108° F.

Neymann and his co-workers (95), similar increased powers of thermo-resistance are exhibited by treponemata inhabiting human lymph glands. They assert that those found in the skin lesions are destroyed by body temperatures of 41° C. maintained for two hours. The same organisms in the glands are not destroyed by temperatures ranging above 41° C. for five or more hours, and above 42° C. for one of these hours.

By holding the tissue containing vaccinia virus at 37.5° C. for varying periods of time between testicular passages in rabbits, Armstrong obtained a virus which showed an increase of several hundred per cent in the time it would withstand a temperature of 37.5° C. Along with the development of this property a marked exaltation of virulence occurred, as was revealed by the increased severity of the testicular lesion and the tendency of the virus to produce lesions in organs distant from the site of inoculation. Castaneda (96) has reported the interesting fact that following X-ray irradiation of guinea pigs, rabbits, and sheep inoculated with the virus of Mexican typhus fever a generalized rickettsial infection of the peritoneum occurs only if the body temperature is kept below 38° C.

From these facts it is evident that various species and strains of organisms may naturally differ in respect to the temperatures at which development may take place *in vitro*, and that this difference can sometimes be correlated with their pathogenic properties for a given animal host. Furthermore, in a suitable environment, changes may occur in the same strain of bacterium or virus in the direction of greater thermo-resistance which go *pari passu* with enhanced virulence.

Presence of Bacteria in Tissues in a Latent Condition. A remarkable instance of bacterial adaptation to the peculiar conditions prevailing in the body at a given time is afforded by the phenomenon of the presence of organisms in tissues without the manifestation of disease. Apparently a delicate local balance is struck between infectious agents and the tissues in which they lie. In such diseases as tuberculosis and syphilis this phenomenon is quite common. The organisms may remain in a definite place for a month and even longer, without giving rise to any signs of disease, and yet, at a given moment, often without apparent cause, a characteristic inflammatory process may be initiated. In the case of experimental syphilis, Zinsser observed a rabbit inoculated with virulent treponema in the testis which showed absolutely no signs of reaction for three and one-half months, at which time a typical process began to appear. Brown and Pearce (97) have found that in experimentally inoculated rabbits the *Treponema pallidum* persists indefinitely in the superficial lymph nodes. The phenomenon has been observed with many different bacteria, even with those ordinarily causing acute disease. We have observed a patient who had recovered from a hemolytic streptococcus infection of the hand. A second operation, made for purposes of improving function at a time when no inflammation whatever existed in the part, revealed the presence of the same hemolytic cocci which had apparently remained latent in the tissues for the entire interval.

The latency of bacteria in tissues is particularly important in the pathology of chronic infections and in such conditions as rheumatism, in which there is a possible allergic basis in the etiology of the disease. In chronic arthritis, in which — with Swift and his collaborators — we favor the allergic point of view, H. Yü and Zinsser (97 a) have found foci of non-hemolyzing streptococci, at autopsy, in heart muscle and in the spleen. It is not unlikely that the spleen is frequently a focus of prolonged latency of this kind and that the condition is particularly frequent when a more or less even balance has been struck between the virulence of the organisms and the susceptibility of the subject. It is of course not clear what the basis of such latency is. Bordet had found that cholera spirilla injected into the blood stream of immune animals may be phagocyted before they are killed by serum bacteriolysis. In his fundamental experiments on experimental streptococcus infection, this author (69) noted that in guinea pigs and rabbits subsidence of symptoms might take place followed by relapse. He suggested that during the period of temporary recovery the organisms might remain viable but quiescent in phagocytic cells. Briscoe had shown that red cells injected into an immunized animal could be phagocyted before hemolysis could take place. Rous and Jones (98), in a series of experiments with *Bacillus typhosus* and guinea pig leucocytes, demonstrated that the organisms may be protected against the destructive action of bactericidal serum after they had been ingested by these leucocytes, which in themselves contain no typhoid-bacillus destructive substances. Rous suggested that bacteria may often be protected from destruction by ingestion within wandering and fixed tissue cells. Fothergill and his co-workers (99) are inclined to believe that the phagocytes offer the same harbor of refuge, as it were, to influenza bacilli in cases of meningitis due to that organism. Such observations suggest a possible site where bacteria might remain for a certain length of time without arousing symptoms of disease.

These examples of the residence of bacteria within tissues without arousing any demonstrable reaction show that the balance struck, as in any infection, must depend to some extent upon the relative speed with which either the invaders or the invaded can modify their biological properties to meet new conditions, Theobald Smith (100, 101) has given this problem most thorough analysis. A microorganism closely adapted to the human body may have developed its offensive and defensive powers to such a degree that when it enters a new human body, it proceeds on its invasive course with speed and violence, and the infected tissues react with a powerful effort to rid themselves of the foreign invader. The result is acute inflammation and disease. As long as the bacteria carry the upper hand, the increase in virulence will continue.

Conversely, however, as the chronicity of the disease is largely dependent upon an adaptation between host and invader in which the reaction to the parasite is less violent, and in which a condition approaching a sort of symbiosis is established. As Smith points out, "there is a struggle on the part of the parasites to adapt themselves and to establish some equilibrium between themselves and their host"; and, again, "the final outcome is a harmless parasitism or some disease of little or no fatality, unless other parasites complicate the invasion."

Such adaptation is not likely to occur unless from the beginning the balance is such that host and invader may be in contact for a period longer than duration of the ordinary acute infectious disease. In cases where the organism is either rapidly gotten rid of, or in which acute death ensues within a short time, such developments can hardly be expected. However, through transitional stages and many successive infections, an originally acute form of disease may become a more subacute or chronic one.

The Problem of Fluctuations in Virulence and Their Relation to Epidemiology. A gradual adaptation of this kind has probably taken place in diseases like tuberculosis, leprosy, and syphilis, in all of which systemic symptoms are manifest only after the disease has gained a more or less extensive foothold. This train of occurrences may also explain the change in what we may call the biology of certain diseases in which there is historical evidence of their great severity in past ages. In the case of syphilis, for instance, its appearance in Europe in the late fifteenth century is recorded as having manifested much greater severity than it does among the same races at the present time. It would seem that in this disease we have an ideal example of adaptation, since the fragility of the organisms and their restriction under natural conditions to a single host prevent their remaining alive outside the human body and in consequence they have passed directly from individual to individual for centuries. But it must not be forgotten that widespread endemic infection of long duration may have also modified in some manner the general herd resistance. Thus it is well known that such diseases as tuberculosis and measles when first introduced among aboriginal peoples, among whom they previously did not exist, cause fulminating infections with very high mortalities.*

The facts concerning the fluctuations of virulence of bacteria as related to host adaptation may have further interesting applications to rise and fall of epidemics. We have long been interested in the curious phenomenon that in epidemics of many diseases, particularly typhus fever, meningitis, and pneumonia, the morbidity and mortality rates are more or less parallel in rise and fall. If microorganisms gain in

* See Chapter IV.

virulence as they are passed from one individual to another, one might expect that, even though the morbidity rate were coming down because of the diminution in susceptible material, the death rate among those infected later in the epidemic would still remain high. It is possible to explain this apparent paradox as follows. Early in the epidemic when a large number of susceptible individuals are available, the organisms pass rapidly from the infected to the healthy. Thus the organisms are transmitted at a time in which they are gaining the ascendancy, and as a result, increasing in virulence. As the epidemic progresses, chances of contact decrease according to a mathematically determinable rate between the diminishing number of susceptibles and the infected.* In consequence the organisms are more apt to enter the new individual later in the disease at a time when the patient has passed his crisis and the bacteria have already begun perhaps to manifest the diminution of virulence which may take place when they are subjected to the presence of defensive serum constituents.

The problem of alterations in virulence during the course of epidemics has lately been subjected to experimental study by Webster and his co-workers in this country, and by Topley and others in England. Employing large colonies of mice into which a naturally infectious agent such as *Bact. Aertrycke* or *Past. murisepticum* had been introduced, the virulence of organisms recovered from the bodies of mice dying as a result of infection was determined from time to time. At first sight it would appear that the results obtained by these two groups of observers would lead to somewhat different conclusions concerning the occurrence of fluctuations of virulence and their role in governing the pattern of any given epidemic. Webster (11), unable to observe that changes in the virulence of the parasite took place in any one epidemic, is inclined to attribute little if any importance to this factor, which he considers to remain practically constant. He recognizes, however, different degrees of virulence as characterizing various strains occurring at different times and in different herds and sees a relationship between this fact and the different courses which these herd infections take (103, 104). Greenwood, Topley, and others (105), however, have obtained data which indicate that changes in virulence of the strain responsible for any specific epidemic may ensue, although this is infrequent. There is some evidence, which is not conclusive, that such changes in virulence occurred with more readiness in the tissues of immune or partially immune hosts than in those of completely susceptible animals. Moreover, these authors have noted alterations in the infectivity, *i.e.*, the power to spread rapidly from individual to individual, of various strains isolated from the same infected herd. This property

* Cf. Zinsser and Wilson (102).

of infectivity, they assert, may be distinguished from virulence since certain strains were found to cause widespread infections, although relatively of low virulence by direct inoculation tests, whereas others were highly virulent but exhibited little tendency to spread. An epidemic may fail to develop in an infected herd because the infecting parasite, although possessing an adequate virulence, lacks infectivity. However, during the natural spread of contact infection an "epidemic" variant may be produced which, combining these two essential characters, induces in a herd a continuous epidemic spread associated with a high mortality. Taking all their observations as a whole, they state that they seem to lend strong experimental support to the view that variations in the infecting agent may be of decisive importance in determining the characteristics of the epidemic process over a long period of time, as well as influencing the behavior of epidemics of the same disease occurring in different places during a relatively short period of time. They do not believe that variation in infectivity and virulence are either essential or frequent concomitants of the evolution of a single epidemic wave, with its characteristic rise and fall. We must confess that when closely examined there seem to be no fundamental divergences between the views of Webster and Topley and his associates. Both have shown that organisms of reduced virulence may be isolated from infected animals, and the latter has emphasized the occurrences of changes in infectivity during the sojourn in the animal body. Neither, however, believes that these changes influence the mortality or morbidity curves of a single epidemic wave to any significant extent, although Topley does not rigorously exclude this possibility. Finally, both freely admit that such variations may account for the characteristics which may distinguish one epidemic from another occurring at a different time and place.

It cannot be candidly maintained that the results of these experimental epidemiologists afford strong support for the hypothesis which we have presented above to explain the parallelism between the rise and fall of mortality and morbidity curves in any given epidemic. Nevertheless, since not only both state that the virulence of an organism infecting a particular animal may vary, but also because such changes have been found by several workers such as Yü (106) and Shibley and Rogers (107) to take place in patients convalescent from diphtheria and in cases of pneumonia, we are inclined to believe that we might go wrong, without additional evidence, in excluding these natural variations of the incitant organisms in their influence on epidemic disease.

Virulence and Dissociation. Investigations carried out during the past twenty years on the phenomenon known as bacterial dissociation or variation have cast considerable light on the biological and chemical

properties which underlie fluctuations of virulence. Bacterial dissociation consists essentially in the appearance, in pure cultures, of organisms which present one or more characteristics by which they may be distinguished from the members of the parent strain. In the case of pathogenic bacteria the parent strain is usually taken as that possessing the attributes exhibited by the organisms when first obtained from a naturally occurring case of the specific infection. To be certain that such variants arise *de novo* from the original strain and do not represent contaminants with similar properties, it is demanded for exacting work that the parent strain shall arise from a single cell isolation. Many observations on variation from the "normal" form recorded in the older literature were no doubt attributable to this process. Metchnikoff and Neufeld, for instance, recognized changes in appearance of growth and relationship to immune serum coincident with loss of virulence.

The paper by Stryker (108) which appeared in 1916 contained a systematic study of the variations induced in virulent cultures of pneumococci grown in type-specific immune serum. Although usually receiving slight notice on the part of most subsequent writers, it is clear that her data establishes many of the fundamental facts concerning this phenomenon and, in our opinion, they should be regarded as a landmark in the vast number of studies which have revealed its great bacteriological and immunological significance. She found that strains of Pneumococcus Types I and II isolated from cases of lobar pneumonia or the peritoneal cavities of infected mice when subjected to action of homologous antiserum (*i.e.*, serum obtained from a horse or rabbit previously injected with the same type of culture) produced variants which differed from the parent strain in respect to (*a*) colony formation, (*b*) the degree to which clumping or agglutination occurred in homologous and heterologous antisera, (*c*) markedly decreased virulence for mice, (*d*) loss of the capacity to form capsules, (*e*) increased ease with which they were taken up by phagocytes in normal serum, (*f*) loss of the property of forming type-specific antibodies when injected into animals, (*g*) failure to absorb type-specific antibodies from homologous immune serum. By repeated animal passage she was able to effect a reconversion of such variants to the original type. The importance of these findings will become more evident to the reader as he gains an insight into the mechanisms of the various immunological processes, but even now it should be apparent from what has already been stated that the way had been prepared for a clearer understanding of those factors which underlie the fluctuations in virulence which appear in response to altered environmental conditions. Following the work of Baerthlein (109), who had emphasized variation in the colony form

of various bacteria and associated these with changes in cultural characteristics and virulence, Arkwright (110) in 1921 considerably extended and clarified our knowledge of the alterations in the appearance of the colony which take place when bacteria dissociate. Studying various organisms of the colon-typhoid-dysentery group, he found that in old cultures two types of colonies appeared which he considered to be of special significance. One he designated as "smooth" because of its glossy, shining surface and even edges, and the other "rough," which was larger with a dull, uneven surface and irregular edges. Transplanted to broth, the "smooth" or S colony cultures grew diffusely, whereas the "rough" or R cultures flaked out and settled to the bottom of the tubes soon after complete growth was attained. In the same year similar observations were made by Schütze (111) and DeKruif (112), again with Gram-negative bacilli. Both of these investigators determined that coincident with the change from S to R colony form a change in serological relationship took place. DeKruif (112, 113, 114), furthermore, showed that the S strains of his cultures of *Past. lepisepticum* maintained their original virulence, while the R variants had become relatively non-virulent. Moreover, an antiserum produced by the injection of the S culture agglutinated both the S and R strains in high dilutions, whereas the serum produced by injection of the R organisms had relatively little agglutinating power for the S culture, although it clumped its homologous bacteria.

In addition to the now well-established R and S variants a third, "mucoid" form is frequently seen. These mucoid phases of various microbial species have long been recognized. Davis (115) in 1912 described a hemolytic streptococcus associated with epidemic milk sore throat which was encapsulated and exhibited large, moist, sticky colonies. More or less analogous variants have been encountered by a number of students of the streptococcus following Davis's original observation. The bacillus of Friedländer and Pneumococcus Type III in their typical forms reveal these mucoid characteristics. Dawson (116, 117) in 1934, having obtained R forms of the pneumococcus which appeared to be even more "rough" than those previously studied, considered these to represent the ultimate R. The former R he designated as S, and the typical, virulent type-specific encapsulated pneumococcus as M. In the majority of cases in which the mucoid variety occurs, it is distinguished by a superior pathogenicity. In the light of these findings it is possible that in the future the normal dissociative process will come to be regarded as including three well-defined, more or less stable phases — M, S, and R. In this book, however, we will for the present adhere to the older terminology. When reference is made to the S form it will signify the variant to which this term was originally

applied. Thus the S form of the pneumococcus indicates the phase characterized by a smooth, shiny colony composed of encapsulated organisms.

Variations in directions other than those which have already been mentioned have been noted by numerous workers,* such as changes in toxin and pigment production, flocculation in various concentrations of electrolytes, resistance to heat and bacteriophage, fluctuations in fermentation of sugars, changes in motility as well as in other morphological respects. Many of these had been observed long before the recognition of the process of dissociation as being a definite attribute of bacterial behavior, but have lately been regarded as particular manifestations of the phenomenon by those writers who have dealt with the subject *a posteriori*. Some of these dissociative characteristics may arise concomitantly with changes in virulence, and the appearance of such variants in a culture may serve as presumptive evidence that a decline in pathogenicity has been inaugurated. It is obvious that decrease in toxin formation could be correlated in this manner. Strains that flocculate readily in physiological saline are generally found to produce disease to a slight degree or not at all in animals, and a similar relationship seems to hold in the case of those organisms showing an increased resistance to bacteriophage. Among motile bacteria variants may arise lacking flagellae which nevertheless appear to retain the same degree of virulence as the parent strain. With pathogenic bacteria, therefore, the dissociative process is frequently but not inevitably accompanied by changes in virulence.

It should be emphasized that the change from S to R as it usually occurs may not always arise as a sudden mutation in which no intermediate stages can be recognized, since the reports of various workers point to the existence of such intermediates, which retain some of the characteristics of the smooth but are definitely proceeding in a direction of the ultimate rough form. In them we should postulate a reduction in pathogenicity without complete extinction of virulence. In the case of the pneumococcus, for example, Blake and Trask (119) and Paul (120) have described a number of intermediates distinguishable by colony form and decreasing virulence as the R variant is approximated. The virulence of a given culture does, however, frequently depend upon a mixture of variants, some of which may possess the maximal degree of infectivity while others are practically avirulent. When introduced into an animal the latter are at once destroyed, while the virulent organisms bring about death, and, when recovered from

* The reader may consult the monograph by Hadley (19) for a full account of such variations up to 1927. More recent literature has been reviewed by the same author (118) in 1937.

the body, appear to have gained in virulence when subsequently tested. This apparent gain is, however, due simply to the removal of the non-pathogenic associates and does not involve changes in the organism itself.*

It was believed for a time that the S to R transformation was irreversible, and that the R form represented a true mutant with stable properties. But it soon became obvious through the work of Griffith (121), Neufeld and Levinthal (122), and Reimann (123) that reconversion of R to S forms could be effected. Griffith showed that when very large quantities of the living R form alone are injected subcutaneously into mice, the animals may succumb after four to five days, and virulent S organisms can be recovered from the local site and the heart's blood. Moreover, if moderate quantities of R culture, alone insufficient to cause death, are injected together with a heat-killed suspension of S organisms, reconversion to the S form is brought about. The type of S pneumococcus thus obtained is determined by the type of killed suspension employed. In this way Griffith succeeded in converting individual R strains into Types I, II, or III irrespective of whether they were originally derived from Type IS, Type IIS, or Type IIIS. Dawson and Sia (124) have succeeded in accomplishing the same result *in vitro* by seeding small amounts of the R culture into suspensions of heat-killed S organisms of a heterologous type contained in a small amount of blood broth and antiserum prepared by injection of rabbits with the R variant. In this manner they changed an R strain derived from Type II into a Type III virulent pneumococcus capable of developing the typical large capsule. In place of the heat-killed suspension, Alloway (125), using cell-free extracts of material obtained from virulent pneumococci, after fragmentation by repeated freezing and thawing, has secured similar reversions to the S form. As will be seen in the next chapter, an essential attribute of virulent pneumococcus is the capacity to produce water-soluble complex polysaccharides which, as was suggested by Zinsser several years ago, appear to form one of the principal constituents of the bacterial capsule. The brilliant experiments of Griffith, Dawson, and Alloway with others that will be subsequently described and which deal with the chemistry of the bacterial cell, demonstrate convincingly that by introducing appropriate modifications in the environment, the metabolic process of the organism leading to the production of a substance upon which its invasive powers largely depend, may be either stimulated, suppressed, or altered so profoundly that it is capable of synthesizing an entirely different chemical compound, but of like significance in respect to pathogenic function. It would seem, however, from the work of

* Cf. DeKruif (114).

Shaffer (126) and his associates, that when an R form derived from a given S strain is reconverted to the smooth form by means of a different heat-killed S suspension, it may not take over precisely the same attributes of virulence as those associated with the organisms used to bring about the transformation. These authors showed that R cocci arising from an S strain of Type III Pneumococcus virulent for mice but not for rabbits, when reconverted in the presence of a vaccine prepared from an S strain virulent for both these animals, proved to possess the same pathogenic properties as the S strain from which it was originally obtained. Conversely, R forms from the rabbit-virulent strain transformed into the smooth state by means of the rabbit avirulent organisms were found to be virulent for rabbits. It was concluded that during the course of dissociation the factors upon which the difference in virulence of the two smooth strains depended were the products of stable physiological processes of the bacterial cell which remained unchanged.

In the realm of the virus agents of disease practically nothing is known concerning processes analogous to dissociation. The fact that fluctuations in virulence occur spontaneously or may be induced by suitable procedures, and that as Craigie (127, 128) and Smith (129) have shown, these entities possess chemical antigenic constituents resembling those found in bacteria might lead us to expect that they would reveal a similar phenomenon. The possibility is strengthened by the recent demonstration by Berry (130) of the transformation of rabbit fibroma virus into the virus of infectious myxomatosis in the presence of material containing the latter inactivated by heat — a finding comparable to that which we have described for the pneumococcus.

The recognition and analysis of the phenomenon of bacterial dissociation have given us a more definite conception of the changes which take place in the bacterial cell when the virulence becomes altered. They have taught us much concerning the conditions which bring about these changes and have brought about an increased realization of the delicate adjustments that exist between the parasite and its surroundings, and the innate instability of a given set of microbial attributes. The important question as to whether dissociation can take place in the body during the course of an infectious disease has been affirmatively answered by such studies as those of Reimann (131), H. Yü (106), and Shibley (107). These observations obviously lead to the thought as to whether artificial measures might not be devised to hasten or to effect this process. Their possible significance in epidemiological problems has already been mentioned in respect to the rise and fall of epidemics. They have, moreover, a direct application to the problem of the management of bacterial carriers, as will be manifest in the chapters which follow. This newer knowledge has already found practical application

in the preparation of more efficient vaccines such as those used in prophylaxis against whooping cough and typhoid fever, and has explained many irregularities in various serological reactions.

Aggressive Substances. It was early suggested by a number of observers that virulent bacteria may secrete substances which promote the further invasion of the tissues. Subsequent experiment has upheld this conception. We know that in addition to the various antigenic constituents of the bacterial cell and the soluble toxins which further the progress of infection, so-called leucocidins (substances which destroy white blood cells) and hemolysins, which dissolve red blood corpuscles, are secreted by bacteria both *in vitro* and in the animal body. In addition, certain organisms such as the staphylococcus produce a substance which clots blood, and the hemolytic streptococcus, a principle that prevents the formation of fibrin. There is, however, at the present time no convincing experimental evidence in favor of an aggressive substance in the sense that this term was employed by Bail (132). This author advanced a theory to explain virulence on the assumption that bacteria only under stress of the struggle against the defenses of the host secreted substances which he called "aggressins." These were not demonstrable in bacterial cultures and were considered in themselves to be entirely non-toxic. Obtained in the exudates from foci of infection such as the peritoneal cavity and freed from bacteria and cells, the aggressins when injected with small doses of homologous bacteria greatly enhanced their virulence. The injection of the aggressin alone was followed by the development of immunity.

These effects can all be obtained with some of the antigenic polysaccharides from bacteria which are readily produced *in vitro* and, it would appear most probable that Bail's aggressins and the substances obtained by Rosenow (133) from pneumococci and termed "virulius" by him, are to be identified with some form of the bacterial antigen.

General Remarks Concerning the Nature of the Infectious Process

Types of Infection. From the facts we have discussed in the preceding paragraphs it becomes clear that the elements which determine the nature of an infectious disease are twofold. On the one hand each variety of infectious germs possesses certain biological and chemical attributes which are specific and peculiar to itself; by these its predilection for path of entrance and mode of attack is determined, and upon these depends the nature of the reaction called forth in the animal body. On the other hand the degree of infection in each case, the severity of the reaction and the ultimate outcome are determined by the balance which is struck between the virulence of the entering germ and the protective mechanism opposed to it.

The specific properties of each microorganism are the factors which account for the clinical uniformity (within definite limits) in the maladies produced in different individuals by the same species of bacteria.

Thus a severe typhoid fever is, in essential characteristics, similar to a mild case — since in both instances the path of entrance, through the intestine, is the same, the distribution after entrance differs only in degree, and the reactions, local and systemic, which are called forth are alike. And cases of this disease in general differ as a class from the maladies caused by, let us say, anthrax infection, where entrance is through the skin, and generalized infection of the blood ensues without definite or regular localization in any given organ. Again, a localized staphylococcus abscess will differ materially from an equally localized focus of tuberculosis, because the chemical constituents of these bacteria respectively call forth each a characteristic response.

Such specificity of the various microorganisms is due partly to their mode of attack and distribution, and partly, as we shall see, to the pharmacological action of their poisonous products.

When these two factors are more or less alike, the clinical types of disease may be similar although the microorganisms causing them may belong to biologically separate species. We need only point out the essential clinical uniformity of the various forms of septicemia, where organisms freely circulate in the blood — with often a focus of distribution on a heart valve — conditions in which it is rarely possible to determine the species of the responsible organism except by blood culture. Or, again, there is great similarity between the ordinary pneumococcus pneumonia and that caused by the Friedländer bacillus. In both cases the distribution and mode of attack of the bacteria are essentially the same, though the microorganisms themselves are biologically dissimilar. The same thing is still more strikingly true of the different types of acute meningitis where lumbar puncture is often the only means of bacteriological diagnosis.

One and the same microorganism, on the other hand, may cause entirely different clinical conditions, as determined by the balance between virulence and resistance. An organism may enter the body and cause an inflammatory reaction at the point of entrance, the process remaining purely localized. In such cases the defensive forces have been so efficient, the invasive properties of the germ so relatively weak, that progression beyond the point of entrance is prevented and the disease takes the form of a localized abscess. Such tendency to localization is the most usual type of staphylococcus infection in which the degree of virulence of the strains ordinarily met is such that the balance struck by them with the average defensive powers of man results in localization. However, the same microorganism, enhanced in virulence, or gaining entrance in unusual numbers in a weakened individual, may rapidly spread, at first by contiguity, then by invasion of the blood and lymph channels, and become generalized.

When organisms become generalized and circulate in the blood, the resulting condition is spoken of as *septicemia* or *bacteriemia*. This is the form of infection commonly caused by streptococci, bacilli of the hemorrhagic septicemia group, anthrax bacilli, and many others. It implies a powerful invasive property and always constitutes a condition of great seriousness. We have learned, however, that in many infectious diseases formerly regarded as purely localized a temporary entrance of the bacteria into the circulation is a usual occurrence. Thus lobar pneumonia is frequently accompanied during the acute stages of the disease by pneumococcus septicemia, and in typhoid fever we know that the organisms circulate freely in the blood during the first two weeks of the disease. The presence of bacteria in the blood is not, therefore, as formerly supposed, an invariably fatal condition.

In these and other conditions the bacteria may be gradually destroyed and disappear from the blood stream as the immunity of the subject increases. In other cases the bacterial activities may be partially checked, the process becoming slower and more chronic. This is especially the case when microorganisms have found lodgment upon a heart valve, from which a continuous supply of bacteria can be given off to the blood. A special form of such "malignant endocarditis" caused by the *Streptococcus viridans* is particularly apt to take this chronic course.

Adami's work indicates that bacteria may normally enter the portal or even the general circulation from the intestine during health. This condition he calls *sub-infection*. That colon and other intestinal bacteria often penetrate into the portal circulation is indicated by the occasional occurrence of colon bacillus abscesses after trauma of the liver.

Significance of Local Reactions. The inflammatory reaction which develops at the point of entrance of bacteria is a result of the local struggle between invader and tissues, and the violence of this reaction is in a large measure an indication of the resistance of the infected subject. When, for instance, a streptococcus gains lodgment in the skin of a resistant individual, the rapid mobilization of leucocytic and other defenses may prevent further invasion by the bacteria and lead to a struggle which is clinically evidenced by severe local symptoms. Did the virulence of the streptococci far overbalance the powers of resistance the local struggle might be reduced to a minimum, the infection progressing without any, or with slight local, reaction. The fact that pneumococci lodging in the human lung ordinarily cause lobar pneumonia is merely an evidence of a considerable degree of resistance to these germs on the part of the average human being. Pneumococci introduced into the pulmonary alveoli of very susceptible animals (rabbits) may pass directly through into the circulation, causing fatal

septicemia without leading to a more than mild and temporary reaction in the lungs themselves. If, as in Wadsworth's (134) experiments, the rabbits are partially immunized — that is, their resistance increased before the pulmonary inoculation is carried out — a violent local reaction, analogous to lobar pneumonia, may follow, the severity of the reaction at the portal of entry being manifestly an evidence of more energetic opposition to further penetration of the bacteria. The innocent appearance of the site of the entrance of the bacteria in generalized streptococcus infection is often nothing more than evidence of the failure of local resistance.

On the other hand, the relatively mild appearance of the initial lesion in syphilis or cutaneous tuberculosis is of opposite significance — in that here it is not a question of the overwhelming of local resistance but signifies adaptation, absence of irritation and slowness of growth on the part of the invader.

Incubation Time. Between the time of entrance of the bacteria into the body and the first appearance of symptoms of disease there is always a definite interval which is spoken of as "incubation time." This period is made up of two definite divisions — one the time necessary for growth, distribution, and accumulation of the bacteria, the other the time necessary for the action of the toxin or poison which may be secreted. The latter, the incubation time of the toxin, is a subject which is still unclear in many of its phases, and will be discussed in a following chapter. The former, however, is to be expected. For the small number of bacteria which gain entrance to the tissues in spontaneous infection is entirely inadequate in itself to produce symptoms. It is necessary that multiplication shall take place until the bacteria have accumulated in number sufficient to cause noticeable disturbance. That the interval necessary for this must vary according to the number of bacteria originally introduced, the virulence of these, and the specific resistance of the patient goes without saying.

But within the limits of the variations introduced by these factors the incubation time of each infectious disease is sufficiently uniform to be characteristic. Thus the primary lesion in syphilis follows the inoculation after an interval of 2 or 3 weeks, rabies follows inoculation with street virus after an average of 28 to 72 days, the period being somewhat dependent on the location of the bite; typhoid fever takes about 2 weeks to develop; gonorrhea about 3 to 7 days; smallpox about 2 weeks; yellow fever 3 to 5 days; and scarlet fever and diphtheria about 2 to 6 days. In general, it may be stated that within the limits observed for each particular infection the shorter the incubation time the more severe is the infection. Thus if tetanus follows inoculation with the tetanus bacillus within 7 days the prognosis is far more

grave than when the incubation time has occupied 2 or 3 weeks. And if localized and general symptoms follow rapidly (within 24 to 48 hours) after a streptococcus infection it is likely that the process is a very severe one.

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CHAPTER II

ANTIGENS

In the foregoing chapter we briefly alluded to the relationship of virulence and the antigenic structure of bacteria, and remarked that along with changes in antigenic structure so frequently observed to take place when organisms undergo dissociation, variation in pathogenicity occurs.

It is our purpose here to describe the most significant properties of these substances spoken of as antigens, that taken as a whole represent one of the two basic factors upon which the processes of immunity depend. For it is indeed toward the elimination or removal of these substances from the body that the operation of the various physiological mechanisms representing collectively that quality of the host which we speak of as "resistance" is directed. Resistance is used here in the widest sense to include the processes not only tending to destroy the parasite or its toxic products, but those which operate to restrict or remove any of the non-living substances which may gain access to the physiological interior of the body and which, because of certain attributes, may be included among the antigens.

Definition of Antigens. An antigen has usually been defined as a substance which gives rise to the development of specifically reacting elements or antibodies in the blood serum or other body fluids of an animal, to which these substances have been administered in a manner excluding their digestive disintegration. The criterion of specificity demands that the antibody react only with the antigen employed in its production or with materials of similar chemical composition. It should be emphasized at this point that the phenomenon of specificity of antigen-antibody reactions is characteristic of many immunological phenomena, and is of the greatest importance in the interpretation of their significance. It will be obvious, however, from what follows that this specificity of antigen-antibody union is by no means absolute. In certain instances overlapping will occur such as is seen with the blood sera of related animal species reacting with the antibody produced by the injection of a single one of them. On first sight what appears to be a still greater divergence from the principle of specificity is manifested in the behavior of the so-called heterogenetic or heterophile antigens which

lead to the formation of antibodies capable of uniting with seemingly entirely unrelated substances. Many of these apparent exceptions, nevertheless, in those instances that have been sufficiently studied, can be explained on the basis of a similar chemical configuration of the antigens.

A further point in this definition requires comment. As a result of the investigations of Landsteiner * upon chemically modified proteins and of heterophile antigens it has been shown that in both these classes (and possibly in certain bacterial antigens) the "complete" antigen (that is, one which can incite antibody formation upon introduction into the tissues of an animal) may be separated into two components. Upon one of these the specificity depends, while the other confers on the complex the capacity to elicit the production of antibody, *i.e.*, renders it a complete antigen. The fraction associated with specificity will react in the test tube with the antibody evoked in response to the injection of the combined form, but alone will not lead to the formation of antibody within the animal body. For this component Landsteiner (2) early suggested the term "haptene." Because of their capacity to react with antibodies and their failure to elicit them, haptenes are sometimes referred to as "partial" antigens.

In defining antigens we would also include a statement indicating that an antigen may react with an antibody naturally present in a given species of animal and not called forth in response to an injection of the antigen. Whether or not such "normal" antibodies nearly always arise as a result of chance contacts with the antigen, or whether they may sometimes be the result of physiological processes which, as in the case of the so-called iso-antibodies of the blood group are inherited characteristics, has not in many cases been determined. This question will be examined in greater detail in our discussion of natural immunity.†

In addition to complete antigens and haptenes there is a third class of substances which are distinguished by certain of the properties of antigens since in the body they lead to definite physiological and, within certain limits, specific changes which become manifested when the same or similar substances are again brought in contact with the tissues. The phenomena appearing in certain of the allergies and hypersensitivities to bacteria or their products, such, for example, as those associated with tuberculosis or sensitivity to certain drugs, are occasioned by these materials acting upon the tissue cells in a manner to change their response upon subsequent exposure to the inciting substances. Here, then, we

* A full account of his work with extensive bibliographical data will be found in the recent monograph by Landsteiner (1) on the specificity of serological reactions, which has been one of our principal sources in the compilation of this chapter.

† See Chapter IV.

have entities which arouse specific alterations in the physiology of cells just as the antigens must do in leading to the appearance of antibodies. The chief difference between true antigens and these antigen-like substances lies in the fact that as yet no circulating antibodies reacting with the latter have been demonstrated to which these particular phenomena of hypersensitivity may be attributed. It is not improbable in these cases that antibody or an analogous principle arises in the cell and remains in such close association with the latter that it does not appear in the body fluids. We are strongly inclined to believe that such substances will ultimately be found to represent true antigens, and that bacterial and other allergies, which as yet have not been shown to depend on antigen-antibody union, will eventually be recognized as arising from this fundamental immunological reaction.

Classes of Antigens. Until recently it has almost universally been held that the only class of materials which could act as complete antigens were the proteins. The latest results, however, of chemical studies on the antigenic structure of bacteria long ago inaugurated by Pick but reopened in the early years of the last decade by Zinsser and his associates and Avery, Heidelberger, and Goebel, indicate that certain complex polysaccharides and lipoid-carbohydrate compounds when freed from all detectable protein may lead to the development of antibodies. The majority of students of this subject appear to accept the view that such polysaccharides are themselves the antigenic agents and are not dependent for their effects upon any extremely minute residuum of protein which may remain following the chemical processes employed in their purification.* At the present time we are aware of no convincing

* Objection to including these polysaccharides among the complete antigens has been made on the grounds that the demonstrated capacity to elicit antibody formation is not inherent in the carbohydrates themselves, but that this is due to accompanying minute traces of some other substance, most probably protein. Felton (3) has calculated that a 10 per cent solution of an antigenic carbohydrate which gives a weakly positive biuret (most of the carbohydrate preparations that have been studied are biuret negative) would contain 0.1 mg. of protein in 1.0 cc. Although extremely small quantities of the carbohydrate will immunize, *i.e.*, induce the formation of antibody, he reckons that they nevertheless contain a considerable amount of protein. On the basis of Felton's figures and experimental data, we find that in the smallest immunizing doses of the carbohydrate there could be present 0.000,000,01 mg. protein if the biuret was weakly positive in a 10 per cent solution. Now according to Wells, the smallest dose of crystalline egg albumin which will sensitize a guinea pig to anaphylactic shock is about 0.000,05 mg., which represents approximately 5000 times as much protein as is contained in the smallest antigenic dose of carbohydrate (1.0 cc. of $\frac{1}{10000000000}$ dilution). The anaphylactic test is perhaps the most delicate for determining the antigenicity of small amounts of protein that we possess. It seems, therefore, extremely unlikely that the very small trace of protein which might be present would account for this effect. An additional reason for believing that the antigenic properties of these substances are due to the polysaccharide is afforded by the fact that only comparatively minute amounts are effective (4, 5). If larger quantities are employed, immunity may not result. Since antibody formation

evidence which would lead us to include in the category of complete antigens any substances other than proteins, certain carbohydrates, and carbohydrate-lipoidal complexes.

In contrast to this group comprised of merely two orders of chemical compounds, that of the haptenes is found to be made up of a wide variety of chemical species, provided we assign to it not only naturally occurring examples but also such substances as may be linked artificially to proteins by suitable chemical procedures. Representatives of haptenes range from alcohol-soluble materials of large molecular size to such simple organic compounds as tartaric acid.

Attributes Associated with Antigenic Function. Practically all naturally occurring proteins are antigenic. Long regarded as the sole possessors of this significant function, they have inevitably been subjected to extensive study in an effort to relate the known facts concerning certain of their physical and chemical properties to the attribute of antigenicity. This object has not yet, however, been attained. We cannot with complete assurance point to any particular characteristic or set of properties upon which depends this capacity to stimulate antibody response. It is highly probable, however, that certain of the physico-chemical characteristics of proteins are involved in determining antigenicity. As Wells (6) points out, to be antigenic a protein must be soluble in the body fluids. Any reaction between the cells and an insoluble antigen would be impossible. Furthermore, it is a general principle that antibodies do not usually appear in response to the injection of a protein of the same species or individual. In other words, it must be chemically foreign to the basic tissue and plasma proteins of the animal into which it is injected. Certain exceptions to this rule have been observed which will be mentioned in connection with the discussion of the factors that determine antigenic specificity.

Other properties of proteins such as molecular size, capacity to form colloidal solutions, failure to diffuse through parchment membranes, digestibility by enzymes, and a definite composition of amino acids, most of which are optically active, may be important factors in rendering these substances antigenic.

following injections of a pure protein does not follow this peculiar behavior, at least within these narrow limits, it is logical to conclude that if accompanying protein contaminants were essential factors in producing the immunological response, this would increase *pari passu* with larger dosage of the carbohydrate.

It has also been urged that the apparently complete carbohydrate antigens become so only after union with the protein of the animal body. That certain exceedingly simple compounds such as formaldehyde and nitrochlorobenzene may, following combination with the body proteins, become antigenic has been demonstrated by the experiments of Horsfall and Landsteiner. Admittedly it is impossible to decide at present whether or not it is by an analogous mechanism that complete antigenicity is conferred upon those polysaccharides which exhibit this property.

The likelihood that large molecular size associated with colloidal properties and inability to pass through membranes plays a role of considerable importance is supported by the studies of Wells and others (7, 8, 9, 10) on the gradual diminution of antigenic properties as smaller molecules are produced by hydrolytic cleavage of the proteins. Indeed it is not impossible that teleologically regarded, the size of the molecule is important in antigenic function, chiefly because it renders the proteins non-diffusible. Since diffusible materials can enter the cell and react with the cytoplasm, there would be no particular reason why the cells should be called upon to form antibodies. With non-diffusible foreign substances, antibody production may be regarded as an emergency apparatus by which the cells are enabled to react with these chemical foreign bodies which cannot pass the cell membrane to enter the cytoplasm. Further evidence for this conception seemed to have been afforded by the experiments of various investigators on the so-called "plasteins." These are proteins formed through the synthetic action of proteolytic enzymes acting on proteoses or peptones which are themselves non-antigenic and of smaller molecular size. The plasteins when tested in animals are found to produce antibodies (11, 12, 13, 14, 15). A reinvestigation of the antigenicity plasteins has, however, been recently carried out by Flosdorf and Mudd (15 a) which casts serious doubt as to whether these substances actually act as antigens or whether the antibodies which appear following their injection are due to accompanying impurities in the form of enzyme protein and traces of undenatured but still antigenic protein from the substrates.

The relationship to antigenic function of the remaining attributes of proteins which have been mentioned above is more doubtful. It was observed by several workers (16, 17, 18) that proteins treated with alkali in such concentration as to induce racemization lost their capacity to produce antibodies. Since Dakin and Dudley (19) asserted that a concomitant loss of susceptibility to enzymatic hydrolysis occurred in racemized proteins, it has been suggested that these alterations were causally interdependent (16). However, more recently acquired data (20) indicate that alkali-altered proteins may not be completely resistant to enzymes, while it has also been shown that marked reduction of antigenic activity takes place before racemization is complete (21). Moreover, as Landsteiner (1) suggests, on treatment with alkali, chemical structures significant for antigenic function may be destroyed which are independent of the mechanism involved in enzymatic digestion.

Because of the fact that gelatin — a protein deficient in tyrosine, tryptophane, and possessing only a small amount of phenylalanine — is non-antigenic, Wells has been inclined to confer a special significance upon the presence of aromatic radicals in respect to antigenic capacity

(6). The observation that proteins deficient in other amino acids, particularly those of the dibasic series, are in no wise antigenically impaired lends force to his hypothesis as well as the fact noted by Obermayer and Pick (22) that chemical alteration of the tyrosine radical of proteins by substitution of iodine or nitro groups leads to pronounced changes in antigenic specificity. Against this inference of Wells's is the lack of antigenic function in proteins altered by alkali which none the less retain a full complement of aromatic groups (16). Furthermore, Hopkins and Wormall (23), by coupling gelatin with compounds containing aromatic groups, failed to obtain antibodies. Hooker and Boyd (24), however, who carried out similar experiments, but with different kinds of aromatic radicals, found evidence that in this way antigenicity might be conferred upon gelatin. However, they conclude that their results do not show that the non-antigenicity of gelatin is due solely to its deficiency in aromatic amino acids.

With the exception of the polysaccharides of complex molecular structure, the proteins are distinguished from all other classes of chemical substances by a peculiar and intricate structure, which, theoretically at least, is capable of almost infinite modification leading to the formation of compounds similar in their properties but none the less sufficiently distinct to possess chemical and serological individuality. It is well known that most of the proteins occurring in nature are built up of about twenty amino acids. On this basis it has been calculated that over 2.4×10^{18} compounds could be formed by combining these acids in different ways. This figure does not represent the theoretical number which could be obtained provided the proportion of amino acids in a given protein was varied. It would appear most likely that not only their properties of immunological specificity, which will be analyzed in a following section, but also their capacity to induce the formation of antibody, is dependent upon this complex structure which must necessarily present large numbers of active atomic groupings and combinations at the extensive surface of a large molecule.

Those polysaccharides which behave like complete antigens share with the proteins the attributes which have been described as probably related to antigenicity. Their molecular weight is high, though it does not appear to equal that of the proteins. They are not diffusible through parchment membranes. Like the proteins, some of them have been shown to be hydrolyzable by enzymes, while they are similarly characterized by complex structures built up of molecules of sugars, sugar acids, or these in possible combination with phosphatides or nucleins.* Because of these properties shared equally by the proteins and car-

* For references to the literature and further details concerning their chemistry, see section on the carbohydrates as antigens in this chapter.

bohydrates, Wells stated in 1929 (6), at a time when the proteins only were still generally considered to possess complete antigenicity, that it would not be surprising if antigenic carbohydrates would be discovered.

In spite of a number of reports which have asserted that lipoids * could act as complete antigens, the majority of investigations dealing with this aspect of immunity have failed to present evidence sufficiently convincing to justify the assignment of these substances to this class. A full discussion of the problem will be found in the monograph of Wells. More recent observations concerning the possible antigenic role of lipoids are summarized in the reviews by Landsteiner (1) and Marrack (25). Perhaps the greatest obstacle to a clearer understanding of those immunological reactions which may involve lipoids is the great difficulty of isolating in a pure condition the various materials obtained by alcoholic extraction. Although crude extractives or those partially purified have acted in some cases as complete antigens, further attempts to separate extraneous matter have resulted in products which were greatly reduced or entirely lacking in activity.

Although it has not been established that complete antigenicity is associated with lipoidal substances, it is fairly certain that in combination with proteins they partake of the nature of haptenes. Analyses of the chemical nature of some of the lipoidal haptenes, however, carried out in recent years, show that in several instances the specific character of the haptene depends rather on the presence of a polysaccharide associated with the lipoid. Further details concerning this problem will be presented below.

The Problem of Antigenic Specificity. The Specificity of Native Proteins. It was almost immediately recognized upon the demonstration of the specific nature of the agglutinin and precipitin reactions by Grüber and Durham and Krause in 1896 and 1897, that in some way this property must depend upon chemical factors peculiar to a given antigen. Early expression of this view was enunciated by Ehrlich. In spite of a few who objected, this hypothesis was held for many years by the majority of immunologists as that best fitted to explain the observed phenomena. But because of the lack of suitable methods, experimental verification was delayed, so that even in 1917, as Landsteiner notes, Morgenroth, a disciple of Ehrlich, could still remark that serology was a field to which there leads no bridge from chemistry. In reality, though, a considerable body of fact had been assembled which

* The term *lipoid* has been used in immunological literature in its broadest sense. Thus it includes the simple lipoids such as fats; the compound lipoids, such as the phosphatides and cerebrosides; and the derived lipoids or those substances obtained from the simple and compound lipoids by hydrolysis, *e.g.*, the sterols and fatty acids.

pointed clearly to a chemical basis for the amazingly delicate specificity of many of the antigen-antibody reactions.

It was primarily the careful and extensive studies of Wells and Osborne (9, 26, 27, 28, 29) and their associates on the naturally occurring proteins in various plant seeds which revealed a close similarity between chemical structure and antigenic function. Begun in 1911, these investigations were continued up to about 1927. They showed, for example, that the alcohol-soluble protein gliadin obtained either from wheat or rye exhibited the same chemical and immunological properties. Hordein from barley — another protein soluble in 70 per cent alcohol, and exhibiting some of the same chemical characteristics as gliadin but not identical with it, was found to give cross reactions by the anaphylactic test with gliadin. However, by desensitization techniques, definite immunological differences could be demonstrated. With the protein known as legumin from the seeds of peas, vetch, lentil and horse bean, which appears to be chemically identical irrespective of its origin, no differences in antigenic specificity were revealed. In contrast, by means of anaphylactic experiments, the exceedingly soluble "protcoses" or prolamines from seeds of the same species were shown to be antigenically distinct from other proteins found in the same seeds. We have already referred to Wells's demonstration of the lack of antigenicity in gelatin which contains no tyrosine, tryptophane, and very little phenylalanine. Other proteins such as gliadin and hordein are also deficient in certain amino acids, notably those containing two amino groups; yet, as Wells and Osborne (26) showed, they were perfectly efficient antigens. Not only does this point to a relationship between chemical structure and immunological activity, but it also suggests that specificity may be to a certain extent conditioned by certain groups or radicals within the molecule.

In addition to the facts revealed by the experiments of Wells, the recognition by several observers, of whom the earliest seems to have been LeBlanc (30), that the various protein fractions of blood serum separable by salting out with ammonium sulfate exhibited distinct specificities when injected into animals, gave further support to the chemical hypothesis. Dale and Hartley (31) were able to distinguish clearly between the albumin, englobulin, and pseudoglobulin prepared from horse serum, and more recently Oguchi (32) reported that by means of the precipitin test he could demonstrate at least seven individual antigens in this serum. Doerr and Berger (33) produced specific antibodies with two fractions of serum albumin obtained at 55 to 66 and 66 to 99 per cent saturation with ammonium sulfate. Reichert and Brown (34) in 1909 had noted that hemoglobin from various species formed, in each case characteristic crystals. The hemoglobin of closely related species gave

crystals which were similar but not identical, but when hemoglobins of distantly related or unrelated animals were examined, the differences were found to be much greater. Not only may the hemoglobins of different species be distinguished morphologically, but they reveal other physicochemical characteristics such as typical absorption spectra (35, 35 a). Following up the observations of earlier authors such as LeBlanc (30) and Thomsen (36), a comparative study of the antigenic properties of various hemoglobins was carried out by Heidelberger and Landsteiner (37) and Hektoen and Schulhof (38) in 1923. As with the crystallographic method, it was found that each hemoglobin was specific for each species, although overlappings occurred between close biological relatives such as the donkey and the horse.

Various other proteins which have well-defined physical and chemical properties are easily shown to be immunologically distinct. Thus the Bence-Jones proteins which occur in the urine in cases of neoplastic invasion of the bone marrow were found to produce specific antibodies which failed to react with normal serum proteins (39, 40). By means of serum reactions Bayne-Jones and Wilson (41) have been able to detect at least two and perhaps three different kinds of Bence-Jones protein. The four proteins of milk, lactalbumin, lactoglobulin, casein, and an alcohol-soluble protein, all possess chemical and serological specificity (42), as well as the several proteins in avian eggs, separable by chemical procedures such as albumin, globulin, ovomucoid, and conalbumin (43).

Nucleoproteins have been especially studied for their antigenic properties by Gideon Wells (44, 45). α-nucleoproteins, which are obtained from tissues by extraction with cold water, salt solution, or weak alkali and then precipitated with acetic acid, are difficult to investigate because of the uncertainty of removal of coagulable proteins. The β-nucleoproteins, however, which are obtained by extractions of tissues with boiling water, and which Wells described as compounds of guanylic acid with protein are much more easily obtained in a relatively pure state and possess definite antigenic properties. Wells suggests the possibility that the nucleoproteins are perhaps associated with the organ specificity which is defined in another place. Pure nucleins are not antigenic.

The examples cited above, as well as many more which have been omitted, justify the conclusion that in general chemical differentiation of proteins can be correlated with antigenic specificity. These studies, however, do not reveal the precise nature of the relationship between chemical structure and specificity.

We are as yet unable to state that a certain group of amino acids arranged in a given way within the protein molecule determine its specific nature. That this is probably the case is suggested by experi-

ments like those of Wells, in which gliadin of wheat and hordein of barley were shown to have a common antigenic factor, while at the same time possessing individual characters by which they could be distinguished anaphylactically. Because of such observations Wells early suggested that the entire protein molecule is probably not involved in determining the specific character of the immunological reactions of proteins, but depends rather on certain groups or radicals. A single protein may, in his view, contain two or more such groups. As we shall see, his conclusions are supported by much evidence secured by other methods.

Species, Individual and Organ Specificity. Not only is there the specificity which we have just seen to be associated with chemically distinct proteins, but a striking specificity exists among many of the proteins of the same chemical group, derived from different species. The classical studies of Nuttall (46) showed that the blood sera of various animal species could in most cases be easily distinguished from each other by means of the precipitin test.* Between the sera of close biological relatives such as man and the anthropoid apes, cross precipitation occurred, *i.e.*, antiserum made by injecting a rabbit with human serum precipitated the sera of the chimpanzee, the gorilla, the ourang, and those of other higher apes. However, in all cases save that of the chimpanzee the reactions were weaker than that with the homologous serum. As the relationship between species becomes more distant, such overlappings disappear. Of course, when the blood serum is employed with its content of several kinds of proteins, antibodies are produced which react with all of these. It has been demonstrated, however, that purified serum globulin or albumin is antigenically characteristic for the species from which it is derived (47). The serum globulin of the horse can easily be differentiated, for example, from that of man, dog, or cattle. Even the albumins of hen and duck eggs, though very similar in most respects, can by proper immunological procedures be identified as distinct proteins (48).

Two other classes of specificity must be mentioned. The fundamental researches of Landsteiner (49, 49 a) on the human blood groups showed that specific antigenic differences existed between the red blood corpuscles of different individuals belonging to the same species. These so-called iso-antigens and their corresponding antibodies were also described at about the same time by Ehrlich and Morgenroth (50), who were able in certain cases to induce antibody formation by injecting the red blood cells of one goat into another. Antigen-antibody reactions of this kind have been accorded extended treatment in a separate chapter.

* Further details of Nuttall's experiments are presented in the chapter on Agglutinins and Precipitins.

In contrast to the more or less narrow species specificity of certain proteins and the sharp differences in iso-antigens among members of the same species is the almost complete lack of specific antigenicity which a few substances of animal origin exhibit. Uhlenhuth (51) early found that the antibody formed in response to the protein of the crystalline lens of the eye reacted with lens material obtained from many species of animals. Nevertheless, even in this case one can recognize a certain degree of specificity, since Hektoen and Schulhof (52) have noted that the lens substance of fish is different from that of mammals. Similar in behavior to lens protein is that of keratin, as shown by Krusius (53), and of thyreoglobulin, which appears to be the same in mammals according to Hektoen and Schulhof (54), but is different in birds. Mammalian fibrinogens are closely related antigenically (54). Some of the materials extracted from various mammalian organs, notably the brain (55), the testis (56), and the placenta (57), appear to have the same antigenic properties regardless of their species source. Antibody-producing substances of this sort have been referred to as organ specific antigens.*

From what has been said it may seem at first sight that in addition to the specificity attributable to chemical nature, other factors might be concerned in that of the species or individual. What evidence we possess, however, indicates that no qualitative differences exist between these classes. There can be little doubt that the chemistry of two globulins from diverse species, such as the horse and fowl, are sufficient to account for the immunological differences. Thus Obermayer and Willheim (58, 59) find that the amino index of the protein fraction of horse serum precipitated at 25 to 30 per cent saturation with ammonium sulfate is about 19, while that of the same fraction prepared from goose serum is about 28.5.

$$\text{amino index} = \frac{\text{total N}}{\text{amino N (formol titration)}}$$

No significant difference was found between the amino index of the same fraction from mammalian sera of different species, but Dakin and his associates (48, 60), by determining the percentages of optically active amino acids in the hydrolytic products of various proteins (sheep and cow casein and ovalbumin of ducks and chickens), conclude that these differ significantly. It is assumed that racemization of peptides depends upon keto-enol tautomerism, and in proteins, therefore, only those amino acids would be racemized in which the carboxyl group was in peptide linkage. In the hydrolytic products only the amino acids carrying free

* For further references to studies on organ specificity cf. Landsteiner (1), page 63, and Chapter IV.

carboxyl groups would in consequence presumably be recovered. Dakin's basic assumption has not gone unchallenged, but whatever the mechanism of racemization, it would seem that his results indicate definite structural differences in the proteins examined. Observations such as those of Horsfall and Goodner (61, 62), who noted that anti-pneumococcus horse serum containing the antibody yielded lecithin when extracted with petroleum ether, while on the other hand anti-pneumococcus rabbit serum gave cephalin, reveal distinctions between the sera from different species which, although not indicative of molecular dissimilarity, make it clear that closely associated substances are present which conceivably might act as haptenes in such a manner as to change the specificity. In the case of the iso-antigens of the blood groups (63, 64, 64 a) and the organ specificity of brain and possibly other tissues, the specificity appears to depend on an alcohol-soluble substance, probably haptene in nature.

It is evident that such data as we have presented above are too meager to afford a conclusive demonstration of the relationship between the antigenic differences which exist between the same protein as defined by chemical criteria, but derived from different species. Protein chemistry has not yet advanced sufficiently to reveal slight deviations in composition or structure, which in all probability underlie the individual and species specificity demonstrable by immunological procedures. The facts, nevertheless, which have been established through the immunological analysis of chemically modified proteins, furnish supportive evidence of a most definite nature.

The Specificity of Chemically Modified Proteins. During the years from 1903 to 1906, Obermayer and Pick (22, 65, 66) carried out a series of investigations which had as their object an analysis of the immunological behavior of various proteins which had been altered by a variety of physical and chemical agents. They distinguished between those which may possibly change only the physical state of the protein molecule and others which introduce new chemical atoms or groups. Concerning the effects of heat or simple acids and alkalies, brief mention will be made at the end of this section. For the moment we are concerned with definite changes in chemical structure. When they produced xanthroprotein by treatment with nitric acid or diazotized the protein with nitrous acid, or iodized it by treatment with Lugol's solution, they found that the specificity became altered. Thus, an immune serum obtained by the injection of animals with any one of these modified proteins failed to react, or only reacted to a slight degree with the unmodified serum protein, but gave strong reactions with similarly altered proteins from other species. A nitroprotein prepared from rabbit serum by treating it with concentrated nitric acid leads to the formation of

antibody which reacts with all other nitroproteins, no matter from what species the normal serum protein is derived. The species specificity has therefore given way to a specificity of chemical structure.

Obermayer and Pick considered these modifications to have been caused chiefly by the character and the position of the substituting groups in the aromatic nuclei of the protein molecule. They accordingly suggested that changes in this group are of paramount importance for antigenic specificity. This would agree with Well's hypothesis that the deficiency in aromatic amino acids is responsible for the lack of antigenic properties found in gelatin, as well as fortify the conclusions drawn by Wells and Osborne concerning the importance of chemical structure. Later studies along similar lines by Wormall (67), Landsteiner (68), Jacobs (69, 70), and others indicate that it is rather the alterations in structure of the tyrosine radical than the chemical character of this class of substituent which are of the greatest importance in determining the specificity. Wormall showed that whether the halogen be iodine or bromine made little difference, but the serological alteration depended upon substitution in the 3, 5 position of tyrosine to give diiodo or dibromo tyrosine. Only very slight differences in specificity are apparent when anti-diazo protein serum is precipitated by the homologous antigen or one containing a nitro group.

Although the importance of modifications in the aromatic nuclei of protein for antigenic specificity cannot be doubted, procedures which leave these radicals undisturbed will nevertheless bring about profound changes in serological behavior. By esterification of the acid groups of the protein molecule as well as methylization of hydroxyl, amino, and imino groups, Landsteiner (68, 71, 72) was able to alter radically the specificity. These esterified proteins behave like the xantho- or iodoproteins in that their capacity to react with antisera for the normal protein is lost, while they cross-react with a large variety of proteins into which the same chemical groups have been introduced. Formolinization of proteins — a process which also presumably leaves unaltered the cyclic groups — brings about analogous specific changes in serum proteins (73).

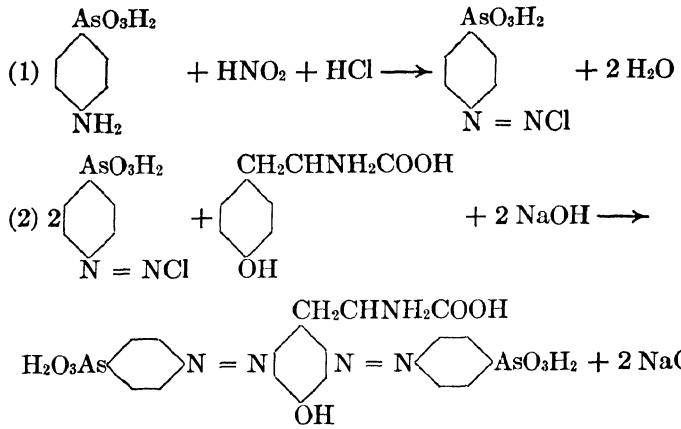
The great significance of special groups and their spatial arrangements in the determination of specificity has been revealed in the extensive investigations of Landsteiner and his co-workers on modified proteins. It is quite impossible within the confines of this book to review in detail all the important work which they have published on this subject.* We must, therefore, select from their experiments a few illustrations of the principles which have been revealed through these studies.

* Landsteiner's Monograph gives complete bibliographical data as well as a summary of his work.

By diazotizing various organic compounds containing cyclic groups — (most frequently the benzene ring) — and then adding these to protein in alkaline solution, Landsteiner obtained a large series of colored compounds which he calls "azoproteins."

Since diazonium compounds in alkaline solution have been shown to combine with phenols, aromatic amines and certain cyclic compounds containing nitrogen in the ring, presumably the diazotized substances under the conditions of Landsteiner's experiments unite with the tyrosine and histidine, and possibly other radicals of the protein molecule * through a diazo linkage.

The fundamental chemical reactions involved are illustrated in the case of the coupling of p-amino-phenyl arsinic acid (atoxyl) to tyrosine:



An antiserum prepared by injecting atoxyl coupled with the protein of normal horse serum will react with any other azoprotein containing this "determinant" group, regardless of the source or kind of protein employed in its preparation. The original specificity of the protein has, then, been changed and is now largely determined by the artificially

* Pauli (74) and Inouye (75) concluded on the basis of experiments with amino acids that diazotized amines couple with proteins only through the tyrosine and histidine groups. More recent findings, however, clearly indicate that the number of these groups present in protein cannot adequately account for the quantity of a given diazonium compound which may be coupled. Thus Boyd and Hooker (76) and Boyd and Mover (77) using diazotized arsanilic acid coupled to protein, found that the ratio of As to N was too high on the assumption that the diazonium salt reacted only with the tyrosyl and histidyl groups. Eagle and Vickers (78) have shown that diazo sulfanilic acid will couple with aliphatic amino groups, the indole of tryptophane and the NH group of proline and hydroxyproline. Unless the reactivity of these radicals is abolished by their incorporation in the protein molecule, it follows, according to these authors, that the reaction between proteins and diazo compounds is not simply due to the coupling with the histidine imidazole ring and the phenyl group of tyrosine.

introduced radical. This alteration in specificity is strikingly shown by the fact that azoprotein produced from rabbit serum proved antigenic when injected into rabbits, thus exhibiting a change sufficient to render it "foreign" in the immunological sense to the animal protein from which it was derived. But the species specificity is not entirely suppressed, for anti-atoxyl horse protein serum will still produce a precipitate with normal horse serum, although this is usually less intense than with the corresponding azoprotein antigen. To obtain reactions, therefore, which are conditioned solely by the nature of the "determinant" group, the "test" antigen usually consists of an azoprotein in which the protein moiety is widely different from that of the azoprotein used to induce the formation of the antibody. For example, if the azoprotein be an atoxyl horse protein, the antiserum is tested against atoxyl-chicken protein, since the antibody which reacts with normal horse serum will not react with normal chicken serum.

It is apparent that an almost unlimited number of different groups may be substituted in the ring of the diazonium compound which is coupled to the protein molecule. By thus substituting various classes of radicals in different positions and then determining the effect on the specificity, Landsteiner arrived at the following conclusions:

1. The chemical nature of substituted acid groups is of paramount importance in fixing the specificity of the azoprotein. For example, antisera produced by the benzene sulfonic acid azoproteins failed completely to react with phenyl arsenic acid protein antibodies, and only exceptionally with antigens containing carboxylic acid groups.

2. Such non-acid or "neutral" groups as methyl, halogen, methoxyl, and nitro substituted in the benzene ring have much less influence than the acid groups on the specificity of the azoprotein. Thus an antiserum against o-chloroaniline coupled with horse serum will react with a variety of azoprotein antigens such as those coupled with aniline, o-toluidine, o-nitroaniline, p-toluidine, p-bromoaniline, etc. Some evidence of specificity, however, was obtained, particularly in the case of the nitro and methoxyl groups, and the carbonyl group behaved much like the acid radicals in that it yielded antigen which failed to cross-react with azoprotein antisera prepared with proteins linked to halogen, nitro, or methyl groups.

3. Experiments with azoproteins containing acid radicals in addition to indicating the importance of the chemical nature of the group, showed that its position in the ring was of almost equal significance. This is well illustrated by the behavior of ortho, meta, and para aminobenzene sulfonic azoproteins in the presence of an immune serum made by injecting the meta aminobenzene sulfonic protein. The strongest reaction, as would be expected, was obtained with the homologous antigen, that with

the ortho form was definitely less, while a minimal precipitate occurred when the acid radical occupied the para position. Similar effects attributable to position were noted with the antigens containing the so-called neutral groups.

Conclusive proof of the determinative role of spatial relationship in antigenic specificity is presented by the results of experiments in which sterio-isometric substances such as d- and l-para aminobenzoyl phenyl-amino acetic acids and the three isomeric forms of tartaric acid were linked to proteins. From the table given below it will be seen that the reactions between the d and l forms and their respective antisera are sharply specific.

Immune Sera	Antigens from:					
	l-tartaric acid		d-tartaric acid		m-tartaric acid	
	COOH | HOCH | HCOH | COOH		COOH | HCOH | HOCH | COOH		COOH | HCOH | HCOH | COOH	
l-tartaric acid . .	+ + +	+ + ±	±	0	+	±
d-tartaric acid . .	0	0	+ + +	+ + ±	+	±
m-tartaric acid . .	±	±	0	0	+ + +	+ + +

Concentration of antigens 0.05 per cent (first column), 0.01 per cent (second column). Taken from Landsteiner (1).

A slight overlapping occurs with the meso tartaric acid antigen in the presence of d and l antisera which may be attributed to the fact that the d and l forms differ in respect to two asymmetric carbon atoms, whereas the meso form differs from the others by only one asymmetric carbon atom.

A further instance of the decisive effect of spatial arrangement is revealed by Landsteiner's work with peptide azoproteins. A number of dipeptides were prepared, such as glycyl-glycine, glycyl-leucine, leucyl-glycine, and leucyl leucine, which were then nitrobenzoylated, reduced to amino compounds, and diazotized and coupled to protein. Cross-precipitin tests with these antigens and their corresponding antisera were then carried out. The results show that a strict specificity existed between the dipeptides glycyl-glycine and leucyl-leucine. Cross-reactions occurred, however, when one of the amino acids was held in common. These were strongest when this acid occupied the terminal position and carried the free carboxyl group. A clearer understanding of the factors

underlying the varied specificity of natural proteins is afforded by these observations, although as Landsteiner points out, they do not provide a full explanation because of the cross-reactions due to the terminal groups. Possibly by employing more complex peptides, sharper differences in specificity might become evident.

These results as a whole were confirmed and extended by means of the so-called "inhibition reaction." This is carried out by adding to an antiserum prepared by means of an azoprotein the uncoupled azo component. No visible effect follows, but a union takes place between the latter and the antibody, since upon subsequent addition of the complete azoprotein antigen a precipitate fails to develop. The azo component, then, is seen to fall into the class of haptenes because it reacts with an antibody; but of course is incapable of functioning as a complete antigen. The inhibition is specific, since the addition of a chemically different haptene sufficiently distinct in chemical structure does not inhibit precipitation with the homologous antigen.

A single example selected from many tests will make this reaction clear.

Materials Used in the Test

1. Azoprotein produced by coupling p-amino-phenyl arsenic acid to horse protein.
2. Antiserum prepared by injecting the same group coupled to chicken protein.
3. p-amino-phenyl arsenic acid.
4. Benzene sulfonic acid.

1 plus 2	= precipitate
1 plus 3	= no precipitate
1 plus 4	= no precipitate
1 plus 3 plus 2	= no precipitate
1 plus 4 plus 2	= precipitate

Not only do such haptenes inhibit the precipitin test in this manner, but they also specifically prevent the serological reactions of the fixation of complement and anaphylactic shock.

The inhibition of antigen-antibody reactions by these compounds of low molecular weight free of protein provides the final proof that antigenic specificity may depend upon a determinative group independent of other groups and not upon altered physical conditions which might be thought to influence its specific activity after becoming an integral part of the protein molecule.

Alteration of Protein Specificity by Agents Which Lead to Denaturation. Soon after the discovery of the precipitin reaction by Kraus and its application by Nuttall to the study of biological relationships, investigations were undertaken with the object of determining the influence of

heat on both the protein antigen and the antibody. The early work, such as that of Graham Smith, suggested that heat destroyed the capacity of the antigen to precipitate with antiserum, but more refined experimentation using dilutions of the antigen, as did Obermayer and Pick (65), showed that heated proteins induced antibody formation and reacted with these antibodies in the test tube. While the "coctoproteins" failed to react with antiserum against the unheated protein, these authors, as well as Schmidt (79, 80), claimed that the species specificity of heated proteins remained unaltered. Subsequent studies, however, make it evident that a definite change occurs in the direction of the broadening of the species specificity. Furth (81), for example, found that an antiserum obtained by injecting beef serum heated at 100° C. reacted with the heated sera of man, horse, and even the rabbit to a slight extent. No precipitation followed the addition of these sera to antiserum against normal beef serum. These observations are in direct confirmation of the earlier findings of Zinsser and Ostenberg (82, 83). Although Furth failed to stimulate the production in rabbits and guinea pigs of antibodies against the heated serum of the same animal, Uwazumi (84) has reported successful results. Obermayer and Pick regarded the changes in antigenicity induced by heat as depending solely on changed physical conditions within the molecule. It is more likely, however, that chemical modifications — possibly a partial hydrolysis or molecular rearrangement — are responsible. It has been demonstrated by Spiegel-Adolf (85) that these modifications, whatever be their exact nature, are not of a permanent order, since heat-denatured proteins can be converted to the normal form by appropriate procedures, and when the latter is tested, it is found that the original specificity has been regained.

Denaturation of proteins leading to changes in specific behavior may also be effected by exposure to simple acid or alkalies, alcohol (21), and even ultraviolet light (86). Recently Basset, Machebœuf, and Wollman (87) by subjecting horse serum to extremely high pressures (4500 atmospheres) have so altered the proteins that they no longer react anaphylactically in animals sensitized with the native serum. The nature of the changes thus induced is at the present time unknown. Possibly here may be an example of specificity influenced by purely physical rearrangements within the molecule, although the fact that even higher pressures lead to coagulation suggests that the process may be analogous to that brought about by heat.

In recapitulation of the foregoing discussion concerning the proteins as antigens it may be stated that all proteins as they occur in nature have the capacity to arouse the formation of specific antibodies provided they are soluble in the tissue fluids of the animal into which they are injected. With the exception of certain complex polysaccharides, the proteins

alone of all the vast variety of natural or artificial chemical compounds appear to be distinguished by this property of antigenicity. But the factors upon which this peculiar characteristic depends have as yet not been unequivocally determined. It is probable that large molecular size and an intricate pattern of chemical groups at the surface of the molecule are essential to this function. Antigenic specificity is certainly conditioned by the composition of amino acids, their linkages and spatial arrangements within the molecule. The specificity of native proteins can be altered by the artificial introduction of foreign groups into the molecule or by measures which effect denaturation.

Carbohydrate Antigens. The recognition of certain complex polysaccharides as complete or partial antigens is, as we have remarked, of comparatively recent date. The study and analysis of their antigenic and chemical properties have therefore been actively pursued for only about sixteen years. But enough has been learned to leave us in no doubt concerning their great significance for the proper comprehension of many bacteriological and immunological phenomena. Earlier a few very suggestive observations had been made. Ford and his collaborators (88, 89, 90, 91) isolated non-protein substances from *Amanita phalloides* (a poisonous mushroom) and poison ivy which appeared to be glucosides, and which they claimed behaved as complete antigens. Pick (92) demonstrated that material from cultures of typhoid bacilli subjected to tryptic, peptic digestion and boiling was precipitated by anti-typhoid bacillus serum, although exhibiting none of the ordinary tests for the presence of protein. In 1917, Dochez and Avery (93) detected in the blood stream and urine of pneumonia patients a substance that reacted specifically with anti-pneumococcus serum, was not destroyed by boiling or by tryptic digestion, was precipitable from aqueous solution by alcohol, acetone, and colloidal iron, and was not dialyzable. They found the same material in filtrates from young cultures of pneumococci and analogous substances in those from certain other bacteria, and concluded they were products of cell growth rather than of decomposition. On the basis of nitrogen determinations they erroneously concluded that these serological active products were of protein nature or associated with proteins.

In connection with experiments upon the tuberculin reaction, one of us in 1921 (94) reported the presence of a substance exhibiting similar properties in tubercle-bacillus extracts prepared in a manner calculated to exclude a considerable proportion of the bacterial proteins. These materials, like those previously described by Pick and Dochez and Avery, were left in alkaline extracts of the fragmented bacterial bodies after removal of the acid-precipitable nucleoproteins, were alcohol insoluble, heat stable, and reacted *in vitro* with specific antisera, by giving precip-

itation and fixation of alexin. Two years later, Zinsser and Parker (95) found similar reactive substances in extracts of pneumococci, staphylococci, influenza and typhoid bacilli. In spite of energetic attempts no antibodies appeared in response to their injection into animals (96). Further developments of this work by many investigators have shown that materials of this character are present in the case of all bacteria in which suitable efforts have been made to find them.

The chemical nature of these non-protein serologically active materials became more precisely defined through the important analyses of Heidelberger and Avery, reported in 1923 and the following years. Working at first with Pneumococcus Type II (97), they separated from large quantities of eight-day broth cultures a polysaccharide by a method of fractional precipitation with alcohol, acetone, ammonium sulfate, and dialysis. On hydrolysis the carbohydrate yielded 79 per cent reducing sugar, a part of which was identified as glucose. Although the first preparation contained nitrogen, subsequent isolations have been obtained free of this element. The purified polysaccharide precipitated the homologous type-specific antiserum in a dilution of 1 : 5,000,000, and it failed to induce antibody formation in rabbits (98) — characters which seemed to identify it as a haptene. Complex polysaccharides were soon after isolated from autolyzed broth cultures of Pneumococcus Types I and III (99) which proved to be quite different in their chemical properties, such as the nature of the hydrolytic products, percentage of reducing sugars on hydrolysis, percentage of C, H, O, the presence of nitrogen in the Type I substance as an integral part of the molecule, optical rotation, and acid equivalent number. These carbohydrates were characterized by sharp type specificity, precipitating only with their homologous antiserum. They too were designated as haptenes, since in rabbits and mice they failed to produce antibodies. These investigations led to the chemical study of a variety of microorganisms. As a result, serologically active polysaccharides have been isolated from the tubercle bacillus, the bacillus of Friedländer, hemolytic streptococci, meningococci, gonococci, anthrax bacilli, members of the Salmonella group, staphylococci, yeasts, etc.* Many of them are far from being satisfactorily described from the standpoint of the chemist. The study of these compounds is still actively pursued, and accordingly the descriptions which are presented here will no doubt be extended and in certain cases modified with the further development of knowledge.

Because the carbohydrates which were first obtained from bacteria appeared to be partial antigens, Avery and Heidelberger (101) and one

* For references to the literature and brief descriptions of the properties of most of these polysaccharides see the reviews by Mikulaszek (100), Landsteiner (1), and Marrack (25).

of us (96) believed that in the intact bacterial cell they must exist in combination with some other substance — most probably the acid precipitable fraction or nucleoprotein — which rendered the complex capable of antibody production. Through autolysis or the chemical manipulations of the isolation process the haptene-like carbohydrate upon which type specificity depends seemed to be split off from the protein. Various reports, however, soon appeared which suggested that active immunity could be induced in certain animals by injecting soluble substances from pneumococci which, from their properties, appeared to be largely non-protein in nature. Perlzweig (102, 103) and his associates in 1923 noted the development of active immunity in mice against virulent pneumococci after treatment with substances derived from the organisms by ammonium sulfate precipitation, bile autolysis followed by precipitation, and by alcoholic extraction preceded by tryptic digestion. These antigens were thus resistant to the action of proteolytic enzymes and were shown to withstand boiling for five minutes. Their studies were continued, and various other methods of obtaining active soluble antigenic preparations were described. Similar observations were made by Ferry and Fisher (104, 105) working with the same organism. Schiemann and his co-workers (106, 107) isolated type-specific polysaccharides from pneumococci, and in 1927 and the following years, demonstrated their capacity to induce type-specific immunity in mice. Wadsworth and Brown (108) likewise immunized these animals by injections of type-specific carbohydrates which they designated "cellular carbohydrates," since they regarded them as more nearly approaching the state in which these substances exist in the living bacterial cell. Not only because this important difference in immunizing action had thus been shown between the pneumococcus polysaccharides originally isolated by Avery and Heidelberger, but also because certain other discrepancies had been revealed by Enders (109) and Pappenheimer (110) in its reactions with type-specific antisera as contrasted with active materials in pneumococcus autolysates and polysaccharide obtained by avoiding an alkaline reaction throughout the process of isolation, Avery and Goebel (111) in 1933 carried out a reinvestigation of the problem. By avoiding the use of an excess of alkali in purifying the carbohydrate of Pneumococcus Type I these authors obtained a product that in minute doses immunized mice and removed all demonstrable antibody from homologous antiserum — effects which were not characteristic of the original soluble specific substance as first prepared. Upon treatment with alkali and heat, these properties were removed and the resulting material proved in all respects identical with the original substance. Avery and Goebel showed that treatment with alkali split off acetyl groups. They regarded the differences in the immunological behavior

between the acetylated and deacetylated forms as depending on the presence or absence of this radical. Enders and Wu (112), working with the polysaccharide of Type I prepared in a similar manner by Pappenheimer (110), obtained results which confirmed the observations of Avery and Goebel. It was further shown that passive immunization with the serum of mice actively immunized with the polysaccharide could be effected. This indicated that humoral antibodies are formed in these animals in response to the presence of the acetylated carbohydrate. The substance in the hands of Avery and Heidelberger, however, produced no antibodies or immunity in rabbits. This remarkable fact has been further substantiated by Downie (113). Indeed from a number of recent studies * it would seem that certain mammals fall into two classes in respect to their reactions to this material. In man, horse, mouse, cat, and dog, immunity follows its injection; while it remains absent in the rabbit, guinea pig, rat, and sheep. No satisfactory explanation has yet been advanced to account for this curious behavior. The problem of the antigenicity of the pneumococcus carbohydrates is further complicated by the fact that the earlier preparations of Heidelberger, Goebel, and Avery which behaved in all respects as haptenis when tested in animals such as rabbits and mice were found by Francis and Tillett (115) to induce a rise in mouse protective antibodies when injected intradermally into human beings. Their observations have been confirmed by Finland and Sutliff (116), and more recently Francis (117), in a repetition of his experiments, has shown that both acetylated and deacetylated forms of the carbohydrate from Pneumococcus Type I are followed by the appearance of mouse protective substances, whereas even precipitins appear in some cases after the injection of the former type of compound. No satisfactory explanation for this difference in behavior conditioned by the species employed has been presented.

Materials apparently containing no intact proteins have been extracted from bacteria other than the pneumococcus and have been shown to possess the attributes of complete antigens. Pick (92), in his noteworthy study of the antigen fractions of the typhoid bacillus, demonstrated the capacity of the alcohol-insoluble fraction of old broth cultures deprived of protein by treatment with ammonium sulfate to produce precipitins in rabbits. This substance was biuret negative, heat stable, and resisted digestion by proteolytic enzymes. Boivin (118, 119, 120) and his collaborators in a series of papers have described the preparation from the Gaertner bacillus and *B. Aertrycke* of carbohydrate phosphatide compounds, which stimulate the formation of specific precipitins and agglutinins in rabbits. In addition they are characterized by considerable

* The literature has been summarized by Horsfall and Goodner (114).

toxicity for these animals, as well as for mice. In the latter these gluco-lipids produce immunity against the toxic action of the antigen or of the living organisms. They are extremely thermostable, remaining biologically active after 30 minutes at 120° C. in the autoclave. By tryptic digestion and alcoholic precipitation, similar if not identical antigenic polysaccharide-lipoidal (phosphatide) complexes have been obtained from *B. Aertrycke* by Raistrick and Topley (121). These likewise induce an active anti-bacterial and antitoxic immunity associated with the production of specific precipitating and agglutinating antibodies. Employing the same method, these authors and their co-workers (122) have derived from *B. typhosus* immunogenic fractions which contain large proportions of polysaccharide. The early finding of Uhlenhuth (123) that the injection of the plant carbohydrate gum arabic led to antibody formation has recently been confirmed by this author in collaboration with Remy (124). The results of certain experiments reported by Sordelli (125) and by Zozaya (126) suggest that agar-agar, a polysaccharide derived from seaweed, can act as a stimulation of antibodies — at least when it is adsorbed to bacteria or other colloidal particles.

This brief survey of the newer knowledge of the immunological properties of certain of these carbohydrates, most of which have been derived from pathogenic bacteria, leaves little doubt that non-protein substances may act as complete antigens. Although the number of examples is not large, we confidently expect that future investigations not only will reveal many additional instances, but also will probably afford chemical data of great value in elucidating the factors responsible for antigenicity, as well as in enlarging our knowledge concerning the basis of specificity.

At present, nevertheless, a number of immunologically active carbohydrates have been isolated which are characterized by haptenic behavior. Of these certain ones may truly belong to this class of antigen, since in the bacterial cell they may be present in combination with a protein, although as yet there exists no direct evidence for compounds of this sort. Others may well have been so damaged during the more or less severe chemical treatment employed in their isolation that the attributes upon which antibody formation depends have been destroyed just as exposure to strong alkali originally used in the Type I Pneumococcus specific soluble substance was found to remove acetyl groups and impair the antigenicity. Carbohydrate haptenes have been converted into complete antigens by combining them with serum globulins through the diazo linkage. Goebel and Avery (127, 128) prepared an amino benzyl ether of the polysaccharide haptene of Type III Pneumococcus and after diazotization attached it to the globulin of horse serum. This antigen

exhibited all the antigenic attributes of the organisms themselves, since it produced in rabbits agglutinating and precipitating antibodies as well as mouse protective substances. Its immunizing properties differ, therefore, from those of the antigenic acetyl polysaccharide of Type I Pneumococcus. In all probability the latter substance is not identical with the carbohydrate as it exists in the bacterial cell. This probability is further supported by the isolation under conditions which minimized the possibilities of hydrolysis by Chow (129, 130) of a type-specific carbohydrate which exhibits a weak capacity to induce antibody formation in rabbits.

The Chemistry of the Immunologically Active Polysaccharides. Many of the bacterial polysaccharides have not as yet been obtained in sufficient quantity or in a state of purity such as to allow a definitive analysis of their chemical composition. The complexity of the molecular structure has furthermore presented impediments in the way of determining the arrangement of the various simple chemical groups of which they are composed. All of them, of course, contain H, C, O, in varying proportions. Some, such as the soluble substance of Pneumococcus Type I, include nitrogen as an essential molecular constituent. In others phosphorus has been detected. Those which have been sufficiently freed of impurities exhibit characteristic rotation of the plane of polarized light and react with definite quantities of alkali (acid equivalents). Their solutions give strong reactions with the Molisch reagent and prove negative to the usual tests for protein. On hydrolysis they yield varying percentages of reducing sugars and sugar acids. Representative data secured from an analysis of the polysaccharides of Types I, II, and III Pneumococci are given in the accompanying table compiled from data lately presented by Heidelberger (131), who has prepared the three type-specific substances by methods which avoid the use of heat, strong acid, or strong alkali. These preparations differ from earlier isolations in a greater relative viscosity of the solution and in precipitating more antibody protein when mixed with homologous antiserum. Certain of the results obtained from analyses of polysaccharides prepared by the older methods are included for the purpose of comparison.

Polysaccharide	Antibody Nitrogen from Rabbit Antiserum, mg. per 1.0 cc.	Ash as Na, Per Cent	N, Per Cent	$[\alpha]_D$ Degrees	Neutral Equivalent	Acetyl, Per Cent	Uronic Anhydride	Reducing Sugars on Hydrolysis, Per Cent	Relative Viscosity of 0.1% Solution in 0.9% NaCl	Amino Nitrogen, Per Cent
Pneumococcus I New Method . .	0.48	3.42	4.62	+ 278	650	7.1	56	30	1.69	2.0
Pneumococcus I Old Method . .	0.08	0.3	5.12	+ 305	417	3.4	65	nd	nd	2.5
Pneumococcus II New Method . .	0.98	2.3	0.40	+ 54	980	1.3	19.1	95	1.38	
Pneumococcus II Old Method . .	0.70	0.0	0.16	+ 52	1000	1.9			1.04	
Pneumococcus III New Method . .	0.78	6.3	0.22	− 32.5	340	0.6	48.2	84	2.64	
Pneumococcus III Old Method . .	0.33	0.7	0.10	− 38.0	351				1.04	

For additional data and details of methods the reader may consult the earlier papers of Heidelberger and his associates which have been cited above.

The study of the molecular structure of the majority of polysaccharides has not progressed sufficiently to enable us to identify all the various constituent groups, their relationships to each other, and to correlate these with immunological behavior and antigenic specificity. In a few cases, however, more than a good beginning has been made toward the attainment of these ends. The carbohydrate of Pneumococcus III has been most thoroughly studied, since it may be isolated in larger quantities and purified more readily and completely than many other similar substances. It behaves as a strong acid and has a molecular weight between 1000 and 5600, according to Heidelberger (132). It appears to be made up of a chain of aldobionic acid units. The aldobionic acid consists of glucose and glucuronic acid joined by a glucoside link (133). Heidelberger (131) is inclined to regard the polysaccharide as a polymer of aldobionic acid and considers the physical and immunological changes induced by heat which have been mentioned above to be explained most simply as a partial depolymerization. It has been demonstrated that the aldobionic acid alone added to Pneumococcus Type III antiserum inhibits precipitation with the polysaccharide, which indicates that the specificity is conditioned by this group (134).

The constitution of the Type I carbohydrate is more obscure. On the basis of analytical data recently obtained, Heidelberger (131) postulates a trisaccharide containing two molecules of uronic acid and an unidentified substance containing two atoms of nitrogen as the basic unit of the molecule. Part of the uronic acid is galacturonic acid. As we have already pointed out, the acetyl group is present and is correlated with its immunizing and serological properties. The analogous substance of Pneumococcus Type II yields nearly 100 per cent reducing sugar on hydrolysis which is mainly glucose. It is probable, therefore, that the substance is made up almost if not entirely of glucose units. A type-specific carbohydrate separated by Heidelberger and Kendall (135) from Pneumococcus Type IV was found to contain on hydrolysis an amino sugar derivative. The presence of a similar compound, it will be remembered, is characteristic of Type I Pneumococcus and is also found among the hydrolytic products of the species-specific "C" carbohydrate described by Tillett and his associates (136), which occurs in all pneumococci irrespective of type or state of virulence. This latter carbohydrate also contains phosphoric acid as an integral part of the molecule.

Immunologically distinct polysaccharides have been isolated from Friedländer's bacillus, types A, B, and C, by Avery, Goebel, and Heidelberger (137, 138). That from type A, like the Pneumococcus Type III substance, is composed of aldobionic acid units containing glucose and glucuronic acid (139). Friedländer type A polysaccharide does not, however, react with Pneumococcus Type III antiserum possibly because of a different linkage between acid and sugar molecules. But there is sufficient chemical similarity between the carbohydrates of Pneumococcus Type II and Friedländer type B to permit the occurrence of cross reactions with the respective antisera (140).

Certain of the serological types of meningococci have yielded specifically reacting complex carbohydrates. Of these only that from Type I has been obtained in a state which permits of anything like satisfactory chemical analysis. On the basis of its properties Scherp and Rake (141, 142) conclude that it is probably a sodium salt of a polysaccharide acid composed of a nitrogen-containing sugar and phosphoric acid units. The polysaccharide of Type III meningococcus appears to be identical with that found in Type I, while that derived from Type II is distinct from either of these.

The complete non-protein antigens isolated by Boivin (118, 119, 120) and Raistrick and Topley (121, 122) from various species of the salmonella group of organisms are carbohydrate-lipid (phosphatide) complexes easily disrupted into a polysaccharide and lipid fraction upon heating in dilute acid. Boivin finds a varying proportion of different fatty acids

in the lipid portion as well as phosphoric acid. Similar findings are reported by the English authors.

Certain of the polysaccharides elaborated by the tubercle bacillus contain varying amounts of pentoses (143). Notable among this class of sugars is d-arabinose, identified by Laidlaw and Dudley (144) and others (145, 146) in carbohydrate fractions derived from this organism. Anderson (147) has obtained from tubercle bacilli polysaccharide-wax complexes. The carbohydrate split off by alkaline hydrolysis reacts to a high titer with specific antiserum. Not only are such carbohydrates found in bacteria but their presence has also been detected in members of the fungi and in yeasts as well as in the cells of the mammalian body and higher plants. Here too they may constitute important factors in the antigenic constitution and behavior of these substances. Raistrick and Rintoul (148) obtained from *Penicillium luteum* a compound termed by them luteic acid, which proved to be composed of units comprising two molecules of glucose and one of malonic acid. The latter forms an ester with one of the glucose molecules which is joined to the other by a glucosidic link. The serological activity of the carbohydrate was not investigated. From yeast Mueller and Tomcsik (149) prepared an immunologically active carbohydrate gum. On hydrolysis the substance yielded 85 per cent reducing sugar. A further analysis of structure and constitution was not carried out. Working with various species of monilia and a trichophyton, Kesten (150) and his associates isolated polysaccharides from each organism which, although exhibiting a certain degree of serological overlapping, could be shown by absorption technique to possess species specificity. Heidelberger, Goebel, and Avery (151), by alcoholic precipitation and partial acid hydrolysis, obtained a substance from gum arabic which precipitated with antipneumococcus Type II and III sera in high dilution. On hydrolysis of the reactive material galactose was obtained, together with at least two complex sugar acids. This heterogenetic behavior of the polysaccharide is without doubt due to the possession of chemical groups similar to or identical with those found in the pneumococcal carbohydrates, although these have not as yet been exactly determined. A carbohydrate has been isolated by Freudenberg and his co-workers (152, 153) from the urine of men belonging to blood group A.* From horse saliva Landsteiner prepared a substance of similar composition. As indicated by the work of Witebsky (156) and his associates, these polysaccharides, which react with group A sera, in their chemical structure are probably similar in some respects at least to the acetyl carbohydrate of Pneumococcus Type I. Like certain of the bacterial carbohydrates, these polysaccharides yield reducing sugars on hydrolysis

* See Chapter X.

(galactose), nitrogen-containing sugars, and acetyl groups. The so-called heterophile antigen of Forssman, serologically related to the group A antigen of the human blood groups and found in the tissues of certain mammalian species, as well as in various bacteria such as *B. Shigae, B. paratyphosus* and *pneumococcus*, although, as we shall see, long regarded as a protein-lipoidal complex, probably is actually a compound structure composed of protein, carbohydrate, and possibly lipoidal elements.

Although only a small fraction of the experimental work dealing with the immunological and chemical properties of the serologically active carbohydrates has been presented in the foregoing paragraphs, it is evident that they have assumed an importance among the antigens scarcely surpassed by that of the proteins. A number of them appear to function as complete antigens, while others, apparently haptenic in their behavior as isolated by chemical procedures, are the determining group in the specific reaction between antigen and antibody.

Structurally they are composed of various species of simple sugars such as hexoses or pentoses and sugar acids which in some instances may contain amino groups. Other groups such as the acetyl may be present and influence their immunological behavior. The simple sugars and acids in those cases sufficiently studied are built up into the complex molecule through a series of glucosidic linkages. In the pure state these polysaccharides exhibit certain properties by which they may be distinguished; such as the degree to which they rotate the plane of polarized light, their elementary composition, neutral equivalents, and percentage of nitrogen. Studies of their serological behavior have served to relate chemical structure with antigenic specificity, thus furnishing further confirmation of this fundamental relationship which had been revealed by investigations on the proteins.

The significance of the serologically active polysaccharides occurring in many pathogenic bacteria can hardly be overemphasized, since it is by their presence certain species of organisms are largely enabled to resist the defensive mechanisms of the host, as will be shown in subsequent chapters of this book.

Lipoids as Antigens. As we have previously stated, no conclusive evidence for regarding lipoids as instigators of antibody production has been presented, although a considerable mass of experimental data dealing with this problem may be encountered in the literature.* The difficulties in the way of obtaining pure compounds from the materials extracted from animal tissues or bacteria have in many instances prevented the formulation of unequivocal experiments. The conflicting

* For bibliographical data consult Landsteiner (1, 157), Rudy (158), and Buchbinder (159).

results of various workers with ethereal extracts from red blood corpuscles are in all probability due to variations in the amounts of non-lipoidal, most likely protein impurities, present in their solutions. Bang and Forssman (160), whose findings in general were corroborated by Landsteiner and Dautwitz, (161), Takaki (162), and others, produced lytic antibodies for the red blood cells of the ox in rabbits injected with ether-soluble substances obtained from these erythrocytes. In contrast, however, to the quantity of untreated cells necessary to elicit antibodies, the amount of ethereal extract required to induce the same effect was relatively much greater, suggesting the presence of a small amount of contaminating material as the active agent (163). Others, such as Thiele and Embleton (164), with large doses of similar materials were unable subsequently to detect any antibody capable of dissolving the homologous red blood cells. Balls and Korns (165) observed that the lipoids extracted from the stromata of red blood corpuscles failed to bind immune hemolysins *in vitro*. But they did not test the antigenicity of the extracts *in vivo*. Similarly, with lipoidal extracts from bacteria and helminths certain investigators have reported the production of specific antibodies. From the tubercle bacillus Much (166), Meyer (167), and others have derived fat-like substances which seemed to act as complete antigens. Schlemmer (168) claimed comparable results with ethereal extracts of typhoid bacilli, but Fürth and Landsteiner (169) failed to confirm his observations. From extracts of tapeworms Meyer isolated a protein and a lipoid fraction. With the latter, specific sensitization of the guinea pig's skin was accomplished, as well as the production of complement-fixing antibodies, which led Meyer (170, 171) to regard the lipoids of the tapeworm as possessing complete antigenicity. Sachs and Klopstock (172), on the other hand, upon repeating Meyer's experiments found no evidence that these substances were antigenic.

In view of all the foregoing evidence, therefore, we cannot be certain that in crude extracts of either tissues, bacteria, or helminths obtained through the use of organic solvents, the lipoidal elements can function as complete antigens. It seems quite certain, however, that with some known lipoids approaching a high degree of purity no antibodies capable either of fixing complement or flocculating with suitable suspensions have been induced. Wadsworth and his co-workers (173), for example, found that the sera of rabbits repeatedly injected with lecithin and cephalin did not contain antibodies against these purified compounds. Considerable data, on the other hand, have been presented which suggest that supposedly lipoidal materials extracted from tissues by organic solvents, carefully purified preparations of naturally occurring lipoids such as lecithin or cholesterol, and even synthetic lipoids may, when mixed with a foreign protein such as pig's serum, become antigenic and

produce antibodies reacting specifically with the lipoid used for immunization. Investigations of this sort were stimulated by the discovery of Landsteiner and Simms (174) that alcoholic extracts of various tissues containing the so-called Forssman antigen, which will be discussed presently, although not in themselves antigenic or at best only revealing very feeble powers of antibody production, became excellent antigens when mixed with swine serum and injected into rabbits. Using this technique, Sachs and Klopstock showed that mixtures of commercial lecithin or cholesterol and pig serum induced specific antibodies in the rabbit. Various authors have confirmed these findings, but the claim of Sachs and Klopstock (175) that these lipoids were the actual determinants of the altered specificity has been made extremely doubtful by such experiments as those of Levine, Landsteiner, and van der Scheer (176), Plaut and Rudy (177), and Wadsworth and Maltaner (173), who showed that mixtures of lecithin and pig serum failed to produce antibody reacting specifically with that lipoid. Others have corroborated the findings of Sachs and Klopstock in respect to cholesterol, but Wadsworth (178) has recently shown that in all probability these results are not due to the formation of a specific antibody reacting with cholesterol but can be more properly attributed to non-specific changes in the serum of the animal following immunization and to fluctuations in the stability of the cholesterol suspensions occasioned by alterations in the amount of protective serum colloids present in dilutions of the antisera prepared with physiological salt solution. Working with purified cephalin, Wadsworth (173) was also unable to demonstrate any haptenic function of this phosphatide in the presence of foreign serum. Because of these considerations the positive findings reported by Weil and Besser (179, 180) concerning the activity of mixtures of serum and synthetic lecithin, synthetic cholesterol, or di-hydro-cholesterol must be interpreted with caution. With such combinations these authors reported the production of antisera which were highly specific for the lipoid injected.

Heterophile or Heterogenetic Antigens (Forssman antigens). Forssman's (181) work, already mentioned, took its departure from a number of observations made previously which considerably disturbed the conception of the strict specificity of antigenic substances. Brezina (182) had found that the injection of sheep's blood often produced hemolytic sera which were more active for beef than for sheep corpuscles, and a number of other observers made similar observations in regard to hemagglutination. Catherine Collins (183) had obtained agglutinins for a number of strains of the dysentery group of bacteria in a goat and in rabbits which she had injected with cultures of brewer's yeast. Frouin (184) reported that he had obtained hemolysins for dog blood by injecting acetone extracts of egg yolk. Forssman began his work with various

extracts of animal organs. We quote some of the conclusions from his original article:

(1) The injection of emulsions of guinea pig liver, kidney, suprarenals, testicles, and brain into rabbits produces sera containing hemolysins for sheep cells. Guinea pig blood does not incite the same antibodies. The hemolysins so produced are comparable in potency to those produced by the injection of sheep blood.

(2) The hemolysins so produced are dependent upon a sensitizer-alexin mechanism analogous to that which governs ordinary hemolysis.*

(3) They are absorbed by sheep cells like ordinary hemolytic sensitizer.

(4) The hemolysins produced in this way are more specific than the ordinary ones produced by blood injection in that they do not react with ox blood.

Forssman thus established the existence, in the organs of a variety of animals, of true antigens which did not follow the usual laws of species specificity and are, therefore, spoken of as "heterophile" or "heterogenetic" antigens.

Before we enter into a discussion of these antigens, it may be well to outline briefly how the presence of a heterogenetic antigen and a specific antibody can be determined. The criterion of a heterogenetic or heterophile antigen is its capacity to induce antibody formation in animals like rabbits and others that will be enumerated.

The sera of animals treated with heterogenetic antigens will be hemolytic for sheep and goat corpuscles and the responsible antibodies are absorbed by such corpuscles.

The heterogenetic antibodies are similarly absorbed by extracts of organs containing heterogenetic antigen.

The antibodies will flocculate alcoholic extracts of similar organs. The antibodies are toxic when intravenously injected into guinea pigs or other animals containing the antigen.

Subsequent investigations have shown that heterogenetic antigens are widely distributed in the animal kingdom. They are present in the organs and tissues but not in the blood of guinea pigs, horses, mice, and pigeons. They are present in the red blood cells of sheep and of goats but not in the organs. Indeed, it is usually the case that when they are found in the organs they are absent in the blood cells and vice versa, but, as Forssman points out, this cannot be established as a rule, since they occur both in the organs and in the blood in chickens, turtles, cats, and probably in dogs. Schiff and Adelsberger (185) have shown that in human beings they are present in the red blood cells of groups A and AB.

The antigens are absent entirely in rabbits, cattle, pigs, rats, and a number of other animals.

* See Chapter VII.

They are also present in certain bacteria such as members of the paratyphoid group, dysentery bacilli, and pneumococci, and are absent from many other bacteria which have been examined (186).

We have given only a partial list of the distribution of these antigens in the animal kingdom and refer the reader to the extensive table which Forssman (187) has published in his review of the subject.

The heterogenetic hemolysins are distinct from the species specific hemolysins, as can easily be shown by experiments with sheep or goat corpuscles. If whole sheep corpuscles, for instance, are injected into rabbits, the two types of hemolysins are formed. If, however, boiled sheep corpuscles are used for rabbit immunization, only heterogenetic hemolysin will be produced and this can be absorbed out either with boiled sheep corpuscles or with heterogenetic antigen from various organ extracts.

It is true in general that heterogenetic antibodies can be produced only in animals in which there is no heterogenetic antigen in the organs or blood. Forssman, however, cites an exception to this in the observation that while horse and guinea pig kidney will produce no heterogenetic antibodies in guinea pigs, nevertheless boiled sheep cells similarly injected will produce moderate amounts of antibody. This fact, he points out, is interesting not only as an exception to the rule stated but also in showing that there are definite differences between individual heterogenetic antigens. The nature of these differences, however, is not clear, since in spite of the facts above described all three of the substances mentioned will absorb the antibody produced by the boiled sheep cells.

Heterophile antigens differ from many of the ordinary antigenic substances in being extraordinarily heat stable. Doerr and Pick (188) boiled horse kidney extracts for from 6 to 8 hours without destroying their antigenic properties, and sheep blood, according to Forssman, remains antigenic after heating to 150° C. They will, moreover, withstand treatment with alcohol and ether for many days without deterioration.

Of extraordinary interest to antigen study in general is the fact that when materials containing heterophile antigens are extracted with absolute alcohol, the extracted organs will no longer fix the antibody, whereas it is found that the alcohol has taken up the haptophore or fixing substance. This very interesting fact has been particularly studied by Doerr and Pick (188), Georgi (189), and others. Although the alcohol takes out the material which unites with antibody *in vitro*, this substance alone will not produce antibodies when injected into rabbits. These facts form an extraordinarily close analogy to the conditions elsewhere described for the splitting of bacterial antigens. Apparently, alcohol extraction takes out that part of the antigen which has the specific

affinity for the heterophile antibody, but this substance represents only a partial antigen or, as Landsteiner has called it, haptene, which is incapable by itself of inciting antibody formation. Only the whole antigen can produce antibodies. Splitting the antigen in this way naturally encouraged many observers to attempt regeneration and recombination of the two parts. Some success has attended the efforts in this direction of Landsteiner (2) and of Landsteiner and Simms (174). By combining concentrated alcoholic extracts of heterophile organs with the serum of the swine, an animal free of heterophile antigen, and mixing these before injection into a rabbit, heterophile antibodies were produced when neither of the combined parts was alone capable of inciting them.

In the experiments of Landsteiner and Simms it was found that the two constituents, swine serum and alcoholic extract, must be mixed and allowed to stand before injection in order that Forssman antibodies may be produced. Injected separately, no such antibodies appear.

An important question arises in regard to the actual union of the alcoholic extracts and the protein to which they are exposed. Neither of these substances is distinguished by marked chemical activity, and the injection of the combinations produces not only heterophile antibodies but antibodies to the protein itself. The latter observation, of course, can easily be explained by a quantitative excess of the serum in the mixtures. Whether actual combination takes place or some process analogous to adsorption we do not know. It was at first assumed, therefore, that the heterophile antigen consists of two parts — a protein fraction which remains behind upon alcohol extraction, and a lipoidal part which goes into solution in alcohol and other lipoid solvents. Investigations by Landsteiner and Levine (190, 191), however, have thrown some doubt upon the actual lipoidal nature of the combining or haptophore group of the heterophile antigen. These workers extracted organs with alcohol, and allowed them to stand in the ice-chest for various periods, during which a precipitate containing the haptophore group of the antigen came down. This precipitate could be dissolved in chloroform and upon reprecipitation in alcohol yielded a substance, still active, which was insoluble in alcohol, acetone, ether or chloroform, but was soluble in water. The watery solution yielded a reducing substance, possible galactose. Because of the large quantity of this reducing sugar and the analytical figures for carbon, Landsteiner is inclined to regard the Forssman haptene, obtained from animal tissues such as horse kidneys, as in part consisting of a carbohydrate which in all probability is in combination with a lipoid. We have already seen that analogous complexes occur in certain bacteria. Moreover, substances definitely carbohydrate in nature have been isolated from various microorganisms which react with Forssman antibodies. In these cases association with

a lipoid appears to be unnecessary for their activity. A close serological relationship has been established by Schiff and Adelsberger (185, 192) between the Forssman haptenes and the group A antigen of the human blood groups. Although this substance has not been isolated in a state which can be regarded as entirely pure, analysis of active fractions indicates large quantities of reducing sugar and carbon contents compatible with the presence of carbohydrate.

On the basis of the available evidence, then, in respect to the nature of the complete Forssman antigen, we may conclude that it consists of a protein and a haptene-like substance. As it exists in many animal tissues, the latter is probably a combination of lipoid and polysaccharide, whereas in bacteria the carbohydrates independently seem to represent the specific substances (186, 193). It has therefore not been satisfactorily demonstrated that lipoids alone can either function as complete antigens or haptenes.

The Forssman antigen and antibody, as was once believed, does not constitute the only example of heterophile or heterogenetic phenomena. The presence of substances which reveal similar or identical serological attributes in entirely unrelated species has been shown to be not uncommon. This is revealed by the cross reactions which take place between the polysaccharides of various unrelated organisms such as B. Friedländer Type B and Pneumococcus Type II (140), Proteus OX 19, and the Rickettsia of Mexican and European typhus fever (194) as well as those demonstrated by Landsteiner and van der Scheer (195) between alcohol-soluble substances obtained from erythrocytes of animals zoologically as widely separated as the horse and rat or the monkey and the pig.* These various heterogenetic relationships must, of course, as we have repeatedly stated, depend upon similar chemical components and structure.†

The Antigenic Attributes of the Cell. In this chapter we have discussed the various substances which may act as antigens or haptenes from the point of view of individual compounds of more or less well-defined chemical and physical properties. It is entirely apparent, however, that the intact cells, whether of animals, plants, or bacteria, contain a number of antigens differing in their specific immunology properties and chemical composition. Maurice Nicolle was among the first to recognize this multiplicity of cellular antigenic structure and aptly referred to the bacterial cell as representing a "mosaic" of antigens. Thus an animal injected with a pure culture of a virulent pneumococcus will respond by the production of at least four antibodies which can be

* For additional references see Landsteiner (1), pages 57–58.

† See section on the Wassermann Reaction in Chapter VIII for a discussion of the possible importance of heterophile antigen in this reaction.

shown to correspond to the type-specific carbohydrate, the species-specific "C" polysaccharide, the so-called nucleoprotein, and the Forssman antigen. The orientation of these antigens in the cell is of great significance, since it is obvious that the one most superficially located will react most easily with its antibody and will accordingly condition those changes in surface properties which are fundamental in the mechanism of immunity. The antigen at the surface also interposes a barrier between those beneath and their specific antibodies. Because of this blocking or masking effect it is possible, for example, to determine pneumococcal types by means of antisera which in addition to the type-specific antibody contain others capable of reacting with the antigens held in common by all pneumococci irrespective of type.

These species antigens, however, appear to be subjacent to the type-specific polysaccharide of the capsule and are thus effectively insulated by it from any interaction with their homologous antibodies.

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CHAPTER III

TOXINS

In the previous chapter we have considered the various classes of substances which may act as antigens and have indicated certain of their properties upon which antigenicity and specificity may depend. Here we shall be concerned with a special order of soluble substances, many of which are antigenic, and which as a whole are distinguished by their toxic effects upon tissue cells.

When bacteria have gained a foothold anywhere within the animal body, the local and general disturbances which follow, in all but the mildest and most trifling cases, are such that we cannot account for them solely on the basis of mechanical injury.

It may well be that the obstruction of capillaries and lymphatics and the pressure upon parenchyma cells, always incident to inflammatory reactions, contribute materially to local tissue destruction, and thereby indirectly to systemic effects. However, even in diseases like anthrax, in which the body of the victim after death is found flooded throughout with masses of bacteria, these factors cannot fully explain the clinical manifestations. And such cases, indeed, are extreme examples, since, in the large majority of bacterial diseases, the illness resulting in the patient is severe out of all proportion to the extent of the tissue area invaded.

Moreover, all infections, if at all marked, whatever their nature or localization, give rise to fever. With this there occur alterations of the blood picture, either a numerical increase or decrease of white blood cells or a change in the relative proportions of the different kinds of leucocytes — or an anemia caused by the destruction of red cells. There may be degenerative changes in parenchyma cells of organs far removed from the actual site of bacterial lodgment. All these facts indicate that, apart from the localized phenomena, there is at the same time an absorption of poisonous substances emanating from the bacteria.

Ptomains. As soon as cultural methods had been introduced, bacteria were studied with the purpose of throwing light upon this phase of their activity. As a result of such investigations, Selmi (1), in 1885, described basic nitrogenous toxic substances which he obtained from putrefying human cadavers and for which he suggested the designation "ptomains" (from

πτῶμα = dead body.)* These poisons were later more extensively studied by Brieger (3), Gautier (4), and others, and it was at first surmised that the formation of such substances in the infected animal might be held responsible for the toxemic manifestations which accompany bacterial disease. This, as we shall see, is not the case. Ptomains are probably not formed in traceable quantity in the living tissues and are not in any way identical with the specific bacterial poisons which are responsible for the toxemia of infectious diseases. For potent ptomains are found in cultures of many bacteria having absolutely no pathogenic power, while highly pathogenic bacteria may produce few or no ptomains. Ptomain production, moreover, is not specific, since the same ptomains may be elaborated by many different bacteria or by mixtures of bacteria, provided the conditions of nutrient materials and temperature are favorable for growth. We cannot therefore account for bacterial toxemia, in which the poison produced by an individual species is usually characteristic and invariably the same, under varying cultural and environmental conditions, by the formation of ptomains. And even when ptomains develop in culture fluids, their physiological action is quite different from that of the poisons originating from the same microorganisms in the infected subject.

Briefly summarized, therefore, the ptomains are poisons elaborated by all bacteria capable of protein cleavage, if planted on suitable nutrient materials under conditions favoring growth. The matrix of these poisons is the protein nutriment; they are not products of intracellular metabolism specifically characteristic of the bacteria which produce them.

It was held by the early investigations that as a result of the proteolytic activities of bacteria ptomains were formed in foodstuffs which, being then ingested, occasioned the toxic manifestations of food poisoning. With the demonstration in suspected foodstuffs by Gaertner, Durham, and many others of certain members of the Salmonella group of organisms, this conception of the pathogenesis of food-poisoning was largely abandoned. Later investigations, however, particularly those of Barber (5) in the Philippines, and Jordan and his associates (6, 7) in the United States, have shown that various bacteria, notably the staphylococcus, may form poisonous substances in foods which, taken into the gastro-intestinal tract, cause severe vomiting and diarrhea. The chemical nature of the toxins is not known, but at present they are regarded as probably products of the bacteria themselves, rather than nitrogenous compounds derived from the decomposition of the proteins of the medium.

Bacterial Poisons. The true bacterial toxins consist of substances arising from metabolic processes and are either permanently or transiently constituent parts of the cell body. They do not represent compounds formed by enzymatic action on the nutritive media in which the organisms are multiplying. This class of poisons has been divided into two groups: the exotoxins and endotoxins.

Exotoxins may be defined as substances frequently, although not inevitably, characterized by a high degree of toxicity for susceptible tissues, which are given off in the surrounding culture fluid possibly as a secre-

* For a historical outline of our knowledge of these poisons, as well as for a thorough treatment of their nature, see Vaughan and Novy (2), "Cellular Toxins."

tion or excretion. They can be obtained in filtrates free of the bacteria themselves at a time when few of the microorganisms have died or disintegrated. The diphtheria and tetanus bacilli produce typical exotoxins.

Endotoxins, typified by the poisons liberated in old cultures of cholera bacilli, appear to be intracellular constituents of the bacterial body which are not set free during life and therefore cannot be obtained in the filtrates of young actively multiplying cultures. The bodies of such bacteria either dead or alive are in themselves toxic when injected into the animal body, and through autolysis or by mechanical rupture of the organisms followed by aqueous extraction, endotoxic substances may be brought into solution.

With the properties of the diphtheria and tetanus toxins in mind, various criteria, in addition to those already mentioned, have been established upon which a given toxic substance may be assigned to the class of exotoxins. The most important of these is the capacity to stimulate the appearance in the animal body of antibody which specifically neutralizes the toxin quantity for quantity. Such antigenic substances originate not only from bacteria, but occur in plants and animals. The vegetable poisons, ricin, crotin, and abrin, snake venom, and spider poisons all exhibit this function.*

* For reference purposes we include in the following list a number of the substances which have been shown to possess the property of giving rise to neutralizing antibodies or antitoxins.

Bacterial toxins

Toxin of C. diphtheriae — Roux and Yersin, 1889
Toxin of Cl. tetani — Behring and Kitasato, 1890
Toxin of Cl. chauvoei — Grassberger and Schattenfroh, 1904
Toxin of Ps. pyocyanea — Charrin, 1887
Toxin of Cl. botulinum — Kempner, 1897
Toxin of Cl. welchii — Bull and Pritchett, 1917
Toxin of Cl. septique — Roux and Chamberland, 1887
Toxin of Cl. oedematiens — Weinberg and Séquin, 1915
Toxin of Cl. sordelli — Sordelli, 1922
Exotoxin of Bact. shigae — Todd, 1904
Erythrogenic toxin of Streptococcus hemolyticus — Dick and Dick, 1923
Fibrinolysin of Streptococcus hemolyticus — Tillett, 1933
Toxins of Staphylococcus aureus — Neisser and Wechsberg, 1901 — J. T. Parker, 1924
Hemolysins from various bacterial, animal, and vegetable sources
Possibly leucocidins from various bacteria

Animal toxins

Snake venoms — Physalix and Bertrand, 1894; Calmette, 1895
Spider poisons — Sachs, 1902
Scorpion venom

Phytotoxins

Ricin from the castor oil bean — Ehrlich, 1891
Abrin from Jequirity bean — Ehrlich, 1891
Crotin from the Croton seed — Morgenroth, cited by Kraus and Levaditi (*Handbuch der Immunitätsforschung*, 1909)
Robin from Robinia pseudoacacia — Ehrlich, 1891
Phallin or amanitatoxin — Abel and Ford, 1907.

In contrast the endotoxins in general either fail to produce neutralizing antibodies or only lead to serum elements which have a remarkably feeble action. A few M.L.D.* may be neutralized by a given volume of serum, but no amount of antibody will inactivate larger increments of the toxin. This difference has lately been well illustrated by the findings of Boivin and Mesrobeanu (8, 9, 10), who demonstrated the presence of an endotoxic gluco-lipid in *B. dysenteriae* (Shiga). While the specific antiserum for this substance was capable of protecting mice against only about 5 M.L.D., the specific antitoxin may protect against many multiple doses of the exotoxin which this organism also produces. Similarly with the gluco-lipids of *B. aertrycke* and *B. pyocyaneus* the homologous antisera were capable of neutralizing only a few minimum lethal doses.

Many of the exotoxins are thermolabile like the enzymes which they resemble in various respects. Some exotoxins are inactivated by heating for a short time (10–60 min.) at 60° to 70° C., but others such as snake venom, the erythrogenic toxin of the hemolytic streptococcus, and botulinus toxin are destroyed at somewhat higher temperatures (80° C.). When snake venom is mixed with hydrochloric acid according to the method of Morgenroth, the combination may resist boiling. If exotoxins are dried, their resistance to heat is greatly increased — a fact which suggests that they may be of protein nature, since pure proteins such as egg white are much more resistant to denaturation by heat when dehydrated. As we shall see, there is additional and more direct evidence for regarding certain exotoxins as proteins. Endotoxins, on the other hand, are in general more resistant to heat — many of them to a remarkable degree. Thus, the cellular toxin of the meningococcus must be heated at 120° C. for 1 hour, whereas the analogous constituent of the gonococcus is not inactivated even under these extreme conditions. Cholera endotoxin is, however, more labile, approaching the more thermo-resistant exotoxins in this respect.

On the whole, the minimal doses of exotoxins required to kill or otherwise demonstrably injure living tissues are small compared with those of endotoxins. Recently Pappenheimer (11), working with diphtheria toxin produced in a synthetic medium, has obtained a highly purified product which has a M.L.D. for the guinea pig of 0.001 mg. The toxic gluco-lipids of Topley and Raistrick (12, 13, 14), Boivin (10, 15, 16, 17), and Henderson and Morgan (18, 19), which appear to represent endotoxins in the purest form in which they have so far been prepared, kill mice only when injected in amounts of 0.1 mg. or more. Potent crude exotoxins of *Cl. tetani* or *Cl. botulinum* are even more poisonous than diphtheria toxin, since they exhibit M.L.D.'s for the guinea pig in the proximity of 0.0005 cc. and 0.0001 cc. respectively, compared with 0.002 cc. for a strong diphtheria filtrate. But the exotoxins of other

* M.L.D. = minimum lethal dose.

bacterial species such as *Cl. welchii* (M.L.D. 0.2 to 3 cc. for guinea pig) and *Cl. chauvoei* (3 to 5 cc. cause local lesion in guinea pigs) are at best considerably weaker, approaching in this respect the endotoxins of such organisms as cholera or *B. coli.*

The pharmacological action of exotoxins which will be subsequently discussed at greater length is variable, but for any one of them is usually quite definite and characteristic. Thus, the neurotoxin (tetanospasmin) of the tetanus bacillus affects chiefly the motor nerve cells. Diphtheria toxin displays a local necrotizing activity on the surrounding tissue while also injuring heart muscles, adrenals, and causing late paralysis provided the animals live. The erythrogenic toxin of the hemolytic streptococcus appears to injure vascular endothelium and in this way induces the typical rash of scarlet fever. Therefore in many diseases where it is the principal factor, the exotoxin through its effects renders apparent the clinical diagnosis. The symptomatology and pathology resulting from injection of endotoxins do not reveal such distinctive attributes, although certain differences may be observed. From whatever source they are obtained they bring about the death of the animal most often within relatively short periods of time without noticeable manifestations other than increasing weakness attended often by signs of diarrhea. Delafield (20), following up earlier observations, has shown that the toxic glucolipid of *B. aertrycke* causes a marked hyperglycemia soon followed by a fall in the blood sugar. With analogous compounds from other Gram-negative endotoxin-forming bacteria Boivin (16) has not only demonstrated the same phenomenon but has found a significant rise in the lactic acid content of the blood. In this respect, then, these endotoxins from different sources exert the same pharmacological effect.

Definite necrotic lesions have lately been observed by Herter and Rettger (21) in the livers of young chickens and turkeys following the administration of endotoxic substances prepared from *B. aertrycke* according to the methods of Raisbrick and Topley and Boivin. Poisonous substances derived from the cells of H_1 pertussis have been found by Bordet and Gengou (22) and by Teissier (23) and his associates to produce extensive necrotizing processes when injected subcutaneously or into the skin of guinea pigs. Other examples of this sort might be cited which indicate that certain endotoxins may produce severe local tissue destruction — a property which they share with some of the exotoxins such as that of *C. diphtheriae.*

An attribute which has sometimes been cited as characteristic of exotoxins is the occurrence of an incubation time between administration and the appearance of symptoms. Certain exceptions occur, however, as with snake venom and the toxins of *Cl. chauvoei* and *Staphylococcus aureus* in which death may follow within a few minutes after intravenous

administration. With others, while the interval is longer after small doses and shorter following larger ones, the incubation period can never be entirely eliminated. In mice, for instance, the incubation time for tetanus toxin may be decreased from 36 to 12 hours by the injection of 3000 minimum lethal doses, but no matter how great the dose it has never been pushed below 8 or 9 hours. The incubation time is nevertheless not typical of exotoxins since a delay of several hours to one or more days may attend the injection of endotoxins.

There has been much speculation concerning the factors underlying the incubation time. It is of course more or less logical to think that a poison which reaches the vulnerable cells through the circulation and lymph should attain its effective concentration in susceptible cells only after a considerable period. Other hypotheses not entirely satisfactory have been proposed. Richet suggested that the toxin itself may not be potent until acted upon by the body fluids and cells of the recipient. De Waele (24) has based an explanation of the incubation time upon a theory of gradual solution of the toxins in the cell lipoids.

It is clear in certain of the cases where there is little or no latent period that the toxin rapidly injures the cells of a vital tissue such as the heart muscle, as recently demonstrated by Dingle (25) and his co-workers with staphylococcus toxin, or the red blood cells which are dissolved, for example, by the hemolytic action of streptococcus lysins or of cobra venom.

Possibly toxins with latent periods act more slowly because they have a greater selective effect upon tissue cells not so immediately concerned in the maintenance of life. Moreover, it is not improbable that the chemical nature of each exotoxin determines the speed with which it may damage a given cell or enzyme system, just as simpler drugs such as hydrocyanic acid and morphine differ greatly in their rapidity of action.

From what has been said in the foregoing comparison of the properties of exotoxins and endotoxins, it becomes evident that it would be difficult to decide on the basis of most of the criteria described to which class a given poison should be assigned. The looseness of association of the poison with the bacterial body, thermo-resistance, pharmacological action, and toxicity vary widely in respect to the different exotoxins, and in any one of these respects they may be difficult to distinguish from an endotoxin. The single criterion upon which, so far as we are aware, correct classification can be made, depends upon difference in antigenic capacity. It is safe to say that no poisonous substance can be regarded as an exotoxin which fails to induce the formation of antibodies capable of neutralizing it according to the so-called law of multiple proportions. This principle may be stated as follows: if "a" units of toxin will neutralize "b" units of antitoxin, then "na" units of one will neutralize "nb" units of the other.

Chemical Nature of Toxins — Exotoxins. The literature on the purification of exotoxins is extensive and here it would not be profitable to attempt a comprehensive review. Most of these investigations have concerned themselves with diphtheria and tetanus toxins, although certain of the vegetable and animal poisons have been purified to a high degree.

We have seen that exotoxins as a group are distinguished by relative susceptibility to inactivation by heat. All of them which have been examined also lose their toxicity either completely or to a marked extent after exposure to low concentrations of formalin. Treatment with formalin and certain other reagents to be mentioned below convert toxin into what is now generally known as toxoid (anatoxin of Ramon). Toxoids when injected into animals do not injure the tissues in the manner of the toxins from which they are derived, but do lead to the appearance of neutralizing or antitoxic antibodies capable of inactivating the untreated toxin according to the law of definite proportions. In addition they retain the function possessed by the original toxin of flocculating with antitoxic sera when the concentrations of both reagents are suitably adjusted.

Most exotoxins are destroyed by proteolytic enzymes, although that of *Cl. botulinus* (26) and of *Streptococcus hemolyticus* (27) resist the action of trypsin — the former completely, the latter to a considerable degree. Like proteins these substances are easily adsorbed on finely divided inert material such as carbon and aluminum hydroxide. They are precipitated from solution by the salts of heavy metals and by weak acid such as acetic, and may be "salted out" in the manner of proteins by suitable concentrations of ammonium sulfate. On the whole they are rapidly impaired by oxidizing agents, by moderate quantities of hydrogen and hydroxyl ions and exposure to light, particularly the ultraviolet. Of course the extent to which they are injured under these conditions varies with individual toxins.

Thus the effects upon the exotoxins of known chemical and physical agents and enzymes which similarly act upon proteins, suggest that some of these poisons at least are also of the same nature.*

But very little is really known, as yet, of the chemical nature of the bacterial exotoxins. However, beginnings have been made in this direction, particularly in the case of diphtheria toxin. This toxin has now been isolated in what appears to be essentially pure form in two different laboratories by Eaton (31) and by Pappenheimer (11) and diphtheria toxoid in a third laboratory by Boivin(32).† All three inves-

* For details concerning the action of various physical and chemical agents on exotoxin, the reader may consult the comprehensive treatises of Pick and Siberstein (28), Walbum (29), and Glenny (30).

† For a critical review of the earlier work on the concentration and purification of diphtheria toxin the paper by Eaton (31) may be consulted.

tigators agree that the toxin is a labile protein and the products isolated in the separate laboratories appear to be identical, containing between 0.00045 and 0.0005 mg. nitrogen per flocculating unit and 10,000 M.L.D. per milligram.* Both Eaton and Pappenheimer have attempted to characterize their product, and the properties which they have reported are given in the following table. It is significant that these properties agree quite closely, though the toxins were prepared on totally different types of media in the two cases.

CHEMICAL COMPOSITION OF PURIFIED DIPHTHERIA TOXIN

	Eaton	Pappenheimer
M.L.D. per mg.	10,000	10,000
Nitrogen per Lf	0.00045–0.0005 mg.	0.00046
Per cent nitrogen	12–16	16.0
Per cent amino nitrogen	2–2.5	1.1
Per cent sulfur	none, as cystine	0.75
Per cent phosphorus	< 0.5	< 0.05
Per cent tyrosine	+ [a]	9.5
Per cent tryptophane	doubtful [a]	1.4
Per cent arginine	+ [a]	3.8 [c]
Per cent histidine	not done	2.4 [c]
Per cent lysine	not done	5.3 [c]
Specific rotation	− 45°	− 36°
*p*H of isoelectric point	between 4 and 5 [b]	4.1 [d]

[a] Color reaction
[b] Minimum solubility
[c] Unpublished communication
[d] By cataphoresis

Convincing independent evidence for the purity of the above material was furnished by a quantitative study of the toxin-antitoxin flocculation reaction from which it was deduced that diphtheria toxin should contain between 0.00045 and 0.0005 mg. nitrogen per Lf unit. The typical protein nature of diphtheria toxin therefore seems assured. Any treatment causing alteration or breakdown of the protein results in a proportionate decrease in toxicity. Thus the toxin is destroyed by proteolytic enzymes (Brandwijk and Tasman (34)), by acid denaturation, and by heating to moderate temperatures. On the basis of its tryptophane content the toxic protein has a minimum molecular weight of at least 15,000. As yet only about 25 per cent of the amino acids composing the toxic protein have been accounted for, and no clear chemical evidence is available to account for its high toxicity. The work reviewed above does emphasize, however, the amazing potency of the bacterial toxins. It may be readily calculated that the minimum skin test dose of diphtheria toxin in the rabbit is less than 1/10,000,000 of a milligram!

* See chapter on Immunity in Diphtheria for an explanation of these terms.

Work on the isolation of other toxins has not yielded products whose purity is quite so well established as that from the diphtheria bacillus, but in a few instances extremely active preparations have been obtained. Thus Sommer (35) has obtained fractions of botulinus toxin of which as little as 0.0002 mg. is lethal per kilo of mouse. The purified material gave a positive biuret test but other protein tests were reported to be negative. Eaton (36) has fractionated tetanus toxin and obtained a toxin protein-like substance with an M.L.D. of 0.00015–0.0003 mg. per kilo of guinea pig.

Although it can be provisionally agreed that diphtheria toxin is a protein, our data are too limited to permit generalization about the nature of all exotoxins. We must not forget the resistance of some of them to proteolytic enzymes, for example, and such reports as that of Maschmann (37), who has carried tetanus toxin through a series of purification steps and claims that his purest preparations gave no biuret or Millon reaction in 0.1 per cent solution, although the M.L.D. for mice was 0.00004 mg.* and the material contained 13 per cent nitrogen. It is therefore not by any means inconceivable that we may eventually find that some toxins are non-protein, possibly carbohydrate substances.

Before leaving this subject, reference should be made to work on the closely related vegetable poisons and snake venoms. In 1905, Osborne, Mendel, and Harris (38) obtained potent fractions of ricin from the castor bean which were typically protein in nature. The most active preparation was lethal in a dose of 0.0005 mg. per kilo of rabbit. Karrer and his collaborators (39) also concluded that ricin was a protein but were unable to obtain a M.L.D. below 0.003–0.005 mg. per kilo of rabbit. Micheel and his co-workers (40, 41) have isolated potent preparations of the cobra venoms, *Naja flava* (M.L.D. 0.12 mg. per kilo of mouse) and *Naja tripudans* (M.L.D. 0.03 mg. per kilo of mouse). The venom contains a high percentage of sulfur (5 per cent), and its molecular weight as estimated by diffusion lies between 2500 and 4000. The poison may be inactivated by oxidation and reactivated by reduction with cysteine. Evidence is presented which indicates that a thiolactone ring may be involved in this reversible change. Wieland and Konz (42), working with the venom of *Naja tripudans*, isolated fractions with M.L.D. of 0.15 mg. per kilo of mouse which gave strong tests for sulfur.

Factors Underlying Toxoid Formation. As yet, no prosthetic grouping has been found to account for the great toxicity of any of the bacterial toxins. So far as the work has gone with diphtheria toxin, it appears to be a typical protein composed of the usual amino acids, and it would seem

* According to the author's calculations this should be equivalent to an M.L.D. of about 0.0003 mg. per kilo of guinea pig and therefore of the same order of potency as Eaton's material.

highly probable that here again, as is now assumed to be the case with insulin (43), and with pepsin (44), biological activity is dependent on the spatial pattern of the amino acids within the molecule, rather than upon any single grouping.

It has long been known that many bacterial toxins lose their toxicity, irreversibly, on treatment with dilute formaldehyde (45, 46, 46 a), but retain their antigenic properties intact. Formaldehyde is known to combine with free amino groups with the formation of methylene linkages to the nitrogen, and consequently, most workers have postulated that the free amino groups (probably the ϵ-amino groups of lysine) are involved in the detoxification mechanism. This view has been further strengthened by the recent work of Goldie (47) using ketene and of Velluz (48) using ketene and phenyl isocyanate as detoxifying agents. Short exposure to ketene acetylates the free amino groups of pepsin (Herriott and Northrop (49)) and of insulin (Stern (50)). Prolonged treatment of these proteins with ketene results in acetylation of the hydroxyl groups of tyrosine. Similarly, Goldie (47) showed that short treatment of diphtheria toxin with ketene results in a loss of more than 99 per cent of its toxicity, but that longer exposure is necessary before its ability to combine with antitoxin is destroyed. About 50 per cent of the amino groups were blocked in this change. Unfortunately, crude toxin was used in this work and therefore no true quantitative estimate of the number of amino groups involved was possible. An attempt in this direction has been made by Eaton (51), who analyzed his pure preparations for amino nitrogen by the van Slyke method before and after treatment with formalin. Eaton also found about a 50 per cent reduction in amino nitrogen upon conversion to toxoid. Since, however, Eaton's amino-nitrogen values for diphtheria toxin are extremely high for a protein the isoelectric point of which is at *p*H 4.1 (at least double that found by Pappenheimer (11)), and since recent work by Dulière (52) has cast some doubt on the validity of using the van Slyke method for determining whether or not amino groups are blocked by formaldehyde, further investigation along these lines is indicated. It is interesting to note that Eaton finds the Sakaguchi test for arginine is weakened after incubation with formalin.

It should also be mentioned here that very little is understood regarding the action of formaldehyde on known amino acids. The reaction has been studied by Holden and Freeman (53) and by Wadsworth and Pangborn (54) under conditions simulating those of toxoid formation. Of the amino acids examined by these workers, tryptophane, histidine, arginine, and lysine, in that order, reacted most readily with formaldehyde. However, except in the case of tryptophane, none of the reaction products have been identified. Tryptophane, incubated with

formaldehyde at 37° C. readily yields the crystalline acid, 3, 4, 5, 6, tetrahydro-4-carboline-5-carbonic acid (Jacobs and Craig (55), Wadsworth and Pangborn (54), Velluz (56)), but this reaction can hardly occur in the toxic protein where both α-amino- and carboxyl-groups of tryptophane are presumably bound to other amino acids through the peptide linkage.

Numerous other reagents have been found which detoxify crude toxins, but these have not received so detailed a study as the formaldehyde reaction and in general have resulted in a simultaneous loss in antigenic power. Schmidt (57) studied the action of more than 200 organic compounds of diphtheria toxin and found that a few aldehydes were the only ones which destroyed the toxicity while leaving the antigenic properties unimpaired. Velluz (48) has shown that phenyl isocyanate and phenyl isothiocyanate detoxify diphtheria and tetanus toxins. The same worker (58) has shown that 0.05 per cent carbon disulfide readily detoxifies tetanus toxin but is without effect on diphtheria toxin. Detoxification of diphtheria toxin by oxidation without loss of antigenicity has been reported by Maloney and Taylor (59) using dyes such as the chloroindophenols. Iodine has been found likewise to convert toxin to toxoid (60). In Glenny's (30) hands the use of toxin modified by treatment with Gram's iodine has proved convenient in the preliminary immunization of animals when formol toxoid or toxin-antitoxin mixtures have not been immediately available. Recently numerous workers (61, 62, 63) have reported on the action of ascorbic acid (vitamin C) on diphtheria toxin. The action of this substance is of interest since it has been shown that the ascorbic acid content of the adrenals falls to a very low level in guinea pigs treated with this toxin. However, such an enormous excess of ascorbic acid is necessary to modify diphtheria toxin even to a slight degree that any relationship between these two substances *in vivo* cannot be due to a direct effect of interaction.

Summarizing our imperfect knowledge of the chemistry of exotoxins, which has nevertheless been greatly extended during recent years, it may be said that certain of these substances are proteins probably of smaller molecular weight than most other members of the class. So far nothing definite in their composition or structure has been revealed which can account for their toxic properties. But the available evidence strongly suggests that certain amino groups are involved.*

Endotoxins. Our knowledge of the nature of endotoxins is exceedingly imperfect.† In the past they have been regarded as more or less

* We are greatly indebted to Dr. A. M. Pappenheimer, Jr., of the Massachusetts State Antitoxin Laboratory, who has kindly written most of the foregoing paragraphs which deal with the chemical properties of exotoxins.

† Much of the older literature on bacterial endotoxins has been summarized by Zinsser (64), and for the typhoid-paratyphoid group, by Branham (65). To the

related to or identifiable with the bacterial protein itself. This view is compatible with the fact that the preparations in which any attempt at purification has been carried out still contain relatively large amounts of nitrogen. But *a priori* the very definite resistance of the majority to elevated temperatures would seem to render unlikely their inclusion among the coagulable proteins. In another respect most of them differ from the exotoxins in not losing their toxicity upon treatment with formaldehyde.* With the findings of Boivin (10, 15, 16, 17) and Topley and Raistrick (12, 13, 14) concerning the toxic properties of the glucolipid antigens isolated from a variety of Gram-negative bacilli, we are in possession of certain new facts which indicate that some endotoxins at least are not proteins. According to these authors the toxicity of the carbohydrate-lipid complex is greatly decreased or entirely eliminated when the linkage between the two principal components is ruptured by heating in weakly acid solution. The phosphatide thus obtained, however, may be slightly toxic in some cases, whereas the carbohydrate variety is entirely innocuous when injected into the animal body. Evidently maximum potency depends upon maintaining the integrity of the complex.

These non-protein toxic substances when in their complete form will produce specific antibodies which neutralize a few M.L.D.'s of the homologous toxin, but will not, as we have observed above, inactivate multiple doses according to the law of definite proportions which holds for the exotoxins. Following the rupture of the linkage between carbohydrate and lipid, antigenicity is completely lost. Neither moiety has the capacity to induce specific precipitating, agglutinating, or neutralizing antibodies which is exhibited by the intact complex.

The toxic substances of Boivin and Topley and Raistrick exhibit differences in specificity depending upon the species of organisms from which they are derived. This is indicated by their behavior with the weakly neutralizing antiserum and the capacity to induce active immunity against homologous toxin.

In view of these data, it is not unlikely that the endotoxic substances prepared by Malcolm and White (76) from meningococci by extraction with solution of sodium hydroxide might be non-protein in nature since the coagulable protein and bacterial nucleoprotein were removed in

references included in these papers we may add those to the work of Olitsky and his associates (66, 67) and of Waller (68) on *B. dysenteriae Shiga*, of Gordon (69), Murray (70, 71), Petrie (72), and Macgraith (73, 74) on the meningococcus, and that of Evans and Maitland (75) on *H. pertussis*.

* The toxic substances of *H. pertussis*, which we regard as endotoxins, although Teissier and his collaborators (23) have urged that they are exotoxic in nature, are inactivated by heating for one-half hour at 55° C. and are rapidly detoxified by 0.3 per cent formalin according to Evans and Maitland (75).

large measure from their preparations by subsequent treatment with acetic acid and boiling although they still contain about 0.76 mg. N per cc. Unlike the gluco-lipid, the meningococcus endotoxin resisted heating when the *p*H was brought to about *p*H 4 with acetic acid. It proved labile, however, in the presence of hydrochloric acid. This conjecture is supported to a certain extent by the observations of Krestownikowa and Rjachina (77), who believe that the meningococcal toxin is a complex nitrogen-containing carbohydrate, and those of Petrie (72), who demonstrated severe toxic injury in guinea pigs injected with a highly purified polysaccharide prepared from Group I meningococcus by Morgan. The symptoms and postmortem signs were similar to those found following the intracerebral inoculation of a crude filtrate of autolyzed meningococci.

Endotoxic-like substances which are, however, in all probability protein in nature have also been demonstrated in a variety of other bacterial species. For example, Boivin (16) finds that the toxic properties of *S. aertrycke*, *S. Gaertner* and *E. coli* cannot be accounted for solely on the basis of their content of gluco-lipids. He has shown that they also contain proteins which are weakly poisonous and which may be separated from the non-protein endotoxins. The toxic proteins unlike the gluco-lipids are found both in the smooth and rough forms, whereas the latter occur only in the smooth, virulent organisms. Herter and Rettger (21) have recently demonstrated the presence of poisonous substances in the filtrates of 12-day cultures of *E. coli* and *S. aertrycke* which kill rabbits and mice and are easily destroyed by proteolytic ferments as well as by moderate heating. The heat-lability of pertussis toxin and its ready detoxification by formalin suggest that it might be a protein or intimately associated with one.

At present, then, it is apparent that there are definite indications that our knowledge of the chemical attributes of endotoxins which have long remained as a class of ill-defined entities will soon be enlarged and extended. Even now it is clear that some of them are definite non-protein compounds which exhibit sharp immunological specificity. Others probably protein in nature are as yet not at all well defined either from a chemical or immunological standpoint. It is of great theoretical and possibly practical interest that the toxic gluco-lipids, as we have seen in the preceding chapter, are also capable of inducing the formation of specific precipitins, agglutinins, and protective antibodies as well as substances capable of neutralizing to a certain limited degree the poisonous effects of these substances.

In connection with these remarks on the nature of endotoxins, we must briefly consider the possibility of toxic substances arising as a result of splitting of primarily innocuous bacterial proteins by enzyme-like prin-

ciples existing in normal or immune serum. At one time this was held by certain authors such as Friedberger (78, 79, 80) and Thiele and Embleton (81) as a most likely explanation of the mechanism of endotoxic action in general. Vaughan (82) had previously shown that all proteins, bacterial or otherwise, would yield upon hydrolysis with alkalinized alcohol, toxic split products which possessed many of the pharmacological properties of the so-called endotoxins. Following Vaughan, Friedberger succeeded in showing that toxic substances similar in their action to Vaughan's split products are formed when specific precipitates, erythrocytes, and bacteria of various species are subjected to the action of normal or immune sera, and that such poisons were pharmacologically alike and produced with equal ease from pathogenic and non-pathogenic microorganisms. Both Friedberger and Thiele and Embleton assumed that the toxic substances were liberated when any antigen combined with antibody in the presence of complement or alexin. The poisonous products, or as Friedberger called them, "anaphylatoxins," were assumed to arise from cleavage of the proteins in the antigen through a proteolytic action of complement and antibody.

Later investigations * have made it clear that not only bacteria but inert substances such as kaolin or barium sulfate added to serum induce the formation of poisonous substances. Finally it was demonstrated that blood itself became toxic if clotting was interrupted or the colloidal equilibrium was otherwise disturbed. Thus the conclusion which Friedberger drew from his observations could not be supported. These phenomena are discussed in greater detail in the section on bacterial anaphylaxis. In spite of this the possibility exists that there may be parenteral splitting of bacterial proteins either within leucocytes or in the body fluids, with the liberation of toxic substances. But it will be evident from the recent work on the nature of endotoxins cited above that many of these exist as pre-formed constituents of the bacterial body. This was the original conception of Pfeiffer and is still that which is most congruent with the experimental facts.

Additional Types of Bacterial Toxins and Toxin-like Substances. A summary of toxins would not be complete unless we also mention here a type of toxic reaction occurring during certain infections which is dealt with more extensively in the chapter on bacterial anaphylaxis and the tuberculin reaction. In the course of many infections beginning within two weeks or less the animal body becomes hypersensitive to substances diffusing from the focus of infection into the circulation. This is the basis of the tuberculin, mallein, and typhoidin reactions and others of this general nature. It is not unlikely that in prolonged or repeated infections much of the toxemia may be due to the action of these mate-

* See a review of this subject by Zinsser (64), and Chapter XII.

rials which are hardly toxic for the normal animal, but highly poisonous to the tissue of an animal which has become hypersensitive through previous contact with them. This type of allergic injury has been regarded as a possible explanation for the local and general manifestations of rheumatism both by Swift and by Zinsser.

Toxic substances, which are probably not related to the poisons of the bacterial cell body, have been obtained from many pathogenic and some non-pathogenic bacteria, both Gram-negative and Gram-positive, by filtration of young cultures and by filtering washings of agar growths. The pertinent literature prior to 1920 has been summarized by Zinsser (64). Parker (83) in the previous year had demonstrated the presence of substance poisonous for rabbits in young broth cultures of *H. influenzae*. No conclusive evidence has been brought to show that poisons produced by these two methods are not identical. These poisons apparently resemble exotoxins in that they in all probability are not products of extraction. They also have a heat-inactivation range (75°-80° C.) approximately that of the more thermoresistant exotoxins. These poisons termed by Zinsser and Parker "X substances," obtained from streptococci, *H. hemophilus*, *E. typhosus*, and several other species, cause marked symptoms of prostration followed in many instances by death within 1 to 6 hours in rabbits injected intravenously. They all were identical in heat resistance, physiological action, and in producing symptoms in rabbits but not in guinea pigs. No conclusive evidence for their capacity to induce the formation of neutralizing antibodies according to the law of direct proportions has been presented. Accordingly, their nature is uncertain, and until further data are available it is impossible to assign them to any particular class of the recognized toxins.

In 1924 Sanarelli (84) showed that normal rabbits treated intravenously with small doses of *V. cholerae* and injected by the same route 24 hours later with a filtrate from a culture of *E. coli* rapidly died with postmortem findings of hemorrhagic inflammation of the gastrointestinal tract, extreme congestion of the viscera, and acute nephritis. Later, Schwartzman (85, 86) described an analogous hemorrhagic reaction which occurred locally in rabbits at the site of an intradermal injection of a filtrate of typhoid bacilli in response to an intravenous inoculation of the same material after 24 hours.

Although a great deal of investigation concerning the nature of the phenomenon has been carried out since that time, its mechanism is still not entirely clear.* It has been established that it is non-specific in the sense that the material employed in the so-called preparatory injection

* Most of the literature on the phenomenon has been summarized and discussed by Schwartzman (87) in his recent monograph.

(*i.e.*, the first injection) may be entirely different from that used in the "reactivating" dose (*i.e.*, in the second injection which for the optimum effect must be given intravenously).

Since the nature of the lesions which result must be due to severe vascular injury, it would seem highly probable that some sort of toxic substance is involved. Since the majority of active preparatory factors are obtained from bacteria either in the form of filtrate or washings of the bacterial growth on agar, it would also appear likely that these organisms produced a substance which is either primarily toxic for the cells of the blood vessels or induces some change in them which renders them markedly susceptible to injury upon subsequent contact with factors present in the material used in the reactivating or provocative dose. It should be mentioned, however, that preparatory factors have been obtained from other sources such as *Ascaris lumbricoides*, yeasts, and possibly milk, while reactivating substances have been demonstrated not only in a wide variety of bacterial filtrates but also in starch, agar, and mixtures of antigen and antibody such as various proteins and their homologous antisera. Accordingly the active substances in the Schwartzman phenomenon are not confined to bacteria. Their chemistry is still unknown, although within the last two years active work on this problem by Schwartzman and others has been in progress. It has been learned that the active principle from bacterial washings or filtrates is relatively heat stable but may vary in this respect according to the particular mode of preparation of the crude material employed. Its activity is destroyed by prolonged exposure to formalin, although its capacity to induce the formation of neutralizing substances is retained. It is not dialyzable through ordinary membranes. Precipitated from solution by either ammonium sulfate or acid alcohol and dried, the material retains its potency for some time. Schwartzman states that taken as a whole the various investigations thus far reported suggest that the active principles are either protein in nature or perhaps associated with proteins and that they are closely related to various antigenic components of the preparations. It should be recalled, however, that Apitz (88) obtained a strongly active fraction from filtrates of typhoid bacilli which gave a strong Molisch test for carbohydrate and failed to give a biuret reaction.

On the basis of available data, therefore, we are not in a position to draw any final conclusions concerning the chemistry of these Schwartzman factors. In respect to their immunological behavior it is probable that since the preparatory factors give rise to neutralizing principles in the serum of animals they are in most instances at least to be classed among the antigens. But Schwartzman does not regard them as either

belonging among the exotoxins, endotoxins, or the X substances of Parker and Zinsser, although he considers them to be more analogous to the first of these classes of reagents. He does believe, however, that they may be fundamentally involved in eliciting the phenomena of bacterial hypersensitivity. But no conclusive evidence for this view has so far been presented.

Toxins and Enzymes. We have already remarked on the similarity which was emphasized by early investigations between exotoxins and enzymes. Both classes of substances have the capacity to react in minute amounts upon relatively enormous quantities of substrate. Like toxins, the enzymes are easily destroyed by heating at 70° to 80° C. for short periods of time. It was formerly supposed that an important difference between exotoxic and enzymatic reactions was the fact that the toxins are bound or neutralized by the tissues they attack, while the enzyme unites with the substance it acts upon and is released when this reaction is completed without noticeable loss in quantity. The investigations of Northrop (89, 90) have to a large extent eliminated this difference, since he has shown that trypsin is inactivated by the substances formed in its action on proteins when a definite equilibrium is reached. The work of this author together with that of others * on the purification of enzymes carried out during the last twelve years has demonstrated that many of these substances, such as pepsin, trypsin, urease, amylase, chymotrypsin, carboxylpeptidase and the yellow respiratory ferment of Warburg are proteins which may be obtained in characteristic crystalline form. As yet none of the toxins have been crystallized. The purified enzymes are easily denatured by heat (about 60° C.) and are active in very small quantities (0.0001 mg. crystalline pepsin powerfully affects the coagulation of milk). As with the exotoxins there is no direct evidence for the existence of any peculiar prosthetic group in the crystalline enzymes which might account for their activity except in the case of the yellow respiratory ferment.

When purified crystalline enzymes became available, it was possible to re-examine the unsolved problem of the antigenicity of enzymes. The results of earlier workers were discordant. Some indicated that specifically reacting antibodies were found following injection of crude enzymes which had the capacity to inactivate the hydrolytic action of the enzyme on its substrate. References to this older literature are given by Wells (91). In 1931 Kirk and Sumner (92), working with crystalline urease, produced specific precipitating antibodies in the rabbit against this antigen. They confirmed and extended these observations in 1934 (93). Northrop (94) had in the year before the

* For an excellent summary of this work and bibliographical information, consult Northrop's second Harvey Lecture (44).

first report of Kirk and Sumner obtained precipitating antibodies against crystalline swine pepsin. Employing the Dale technique Ten Broeck (95) in 1934 demonstrated specific reactions in the isolated guinea pig uterus* upon the addition of crystalline pig and beef trypsin as well as chymotrypsin and its precursor chymotrypsinogen. In this manner, species differences between the enzymes obtained from beef and swine could also be differentiated. Seastone and Herriott (96) in 1937, working with crystalline pepsin which rapidly undergoes denaturation at about *p*H 7.6 and presumably is thus changed when injected into the animal body, obtained antibodies which precipitated the denaturated pepsins from swine, cattle, and guinea pigs but failed to react with the serum proteins of swine or with pepsins from rabbits, chickens, or sharks. Similarly with pepsinogen antisera, reactions were observed with pepsinogen but not with pepsin or homologous serum proteins. On the basis of these new data secured through the use of purified enzymes, we can be reasonably certain that these substances are antigenic, and therefore do not differ in this respect from the exotoxins as some authors have previously maintained. It is evident then that all the lately acquired information concerning both exotoxins and enzymes serves to reveal an increasing number of attributes possessed in common by both classes of substances.

Mode of Action of Bacterial Poisons. To injure the infected individual, the bacterial poisons must be produced in such locations that they can easily enter the physiological interior of the body and thus locally or by migration to distant sites affect the susceptible tissue cells. As we have noted, few of the poisons are harmful when introduced into the intestinal canal. In this location they are, as a rule, destroyed, or they pass through without effect. Neither diphtheria nor tetanus toxin will produce symptoms when introduced into the intact gastrointestinal tract. Even cholera poison does not appear to pass through the undamaged intestinal wall. Kruse (97) called attention to experiments by Bürgers in which enormous quantities of dead or living *V. cholerae* which contain potent toxins could be administered by mouth to healthy guinea pigs and rabbits without the production of disease of any kind. It is likely that the absorption of poison begins only after the bacteria have invaded the mucosa and, by injuring tissue, have opened paths for absorption. In the case of diphtheria probably a similar condition exists in that the localized injury to the mucous membrane affords a portal for the absorption of the toxin into the general lymphatic and vascular system, whence it reaches vascular endothelium, nerve cells, adrenals, and heart muscle tissues, which are particularly susceptible to its action. The poison of *Cl. botulinum* and the gastro-

* See Chapter XII for the immunological principles underlying this reaction.

enteric toxin of certain staphylococci form exceptions to this rule, since they are absorbed directly from the intestinal canal. Certain snake poisons, especially those of some of the *viperidae*, may also cause a violent gastro-intestinal irritation if taken by mouth, and the vegetable toxins like ricin are characteristically powerful if ingested.

The toxic injury of the intestinal tract occurring in the course of such infections as bacillary dysentery, typhoid fever, and some others is probably not due wholly to the action of the toxins in the intestine, but also to injury produced in the course of their excretion through the intestinal mucous membrane.

Why certain toxins should be innocuous after ingestion, while others are absorbed in an active state is not entirely clear. Certain of the exotoxins are certainly more resistant than others to the action of proteolytic enzymes. This does not necessarily imply, of course, that the resistant ones are not proteins, since it has recently been shown, for example, by Bawden and Pirie (98) and Martin and his associates (99) that the virus protein of the mosaic disease of tobacco withstands the action of trypsin. It is possible, moreover, that some of the normal bacterial flora of the gastro-intestinal tract may exert an antagonistic action on certain poisons. There may also be an antagonistic effect of some of the constituents of the bile.

The action of many bacterial poisons, as we have already remarked, is, like that of alkaloids and other drugs, selective in respect to various tissues. Whereas most of these poisons excite inflammatory reactions, most probably through their effect on the capillaries and small blood vessels, if concentrated in a local area, they also exhibit a specific distribution which shows that they go into selective relationship with certain tissues and cells just as the neurotropic viruses such as rabies, poliomyelitis, and encephalitis reveal a definite affinity for nervous tissue. Many examples of this behavior on the part of toxins might be cited. Thus, the bacterial hemotoxins or hemolysins dissolve red blood cells both *in vitro* and in the infected body and the leucocidins of the staphylococcus and streptococcus injure white blood cells, particularly those of the granulocytic series. Diphtheria toxin, after distribution through the body, may act upon many different tissues, as is evident by degenerations in heart muscles, liver, kidney and suprarenal glands, and the petechial hemorrhages in serous surfaces, as well as by the late paralyses which may develop both in man and in animals. The localization of the diphtheritic lesion may influence the selection of individual nerves, with those most severely affected lying adjacent to or actually within the area involved; for this poison, like tetanus toxin, may be absorbed directly into the nerves through the nerve endings as indicated by the work of Myer and Ransom (100, 101, 102).

One of the most interesting and important effects of diphtheria toxin which have been enumerated is that upon the suprarenal glands. Experimentally in guinea pigs the acute hemorrhagic reaction in these organs is a prominent and diagnostic feature in the pathology of the disease. In human beings, although it has long been recognized that changes may occur in the adrenals in human cases of the disease, they are by no means as regular or severe as in the guinea pigs. Abramow (103), in 1912, studied the lesions occurring in the suprarenals and described them as consisting of a degeneration and final disappearance of the chromaffin substance of the medullary cells. He believed that this, together with the degeneration of the heart muscles, is of importance in causing the characteristic vascular failure which accompanies the terminal stages. Maclean (104) has recently reviewed the literature on this subject, and although in general his conception of the importance of the role played by the adrenals in diphtheria is in agreement with Abramow, he finds a somewhat different pathological picture in the gland. From his own observations in autopsies of fatal cases Maclean concludes that when minimal intoxication occurs such as in membranous croup, the glands show either no visible pathology or only slight hemorrhage between the fasciculi of the zona reticularis of the cortex. When the poisoning is greater, hemorrhages occur in all three layers of the cortex and in the region of the medulla proximal to the cortex. Various degrees of cloudy swelling were seen in the cells of the cortex. In the most severe cases hemorrhages are so marked that they may be observed on the surface of the organ. These findings are in accord with the early conclusions of Meyer and Gottlieb (105), who classed diphtheria toxin as a specific vascular poison, since in this organ at least there is definite indication of damage to the capillary walls. Maclean, on the basis of his clinical and pathological studies, states that there is a large amount of evidence in favor of the view that lesions of these glands play a considerable part in producing the typical symptomatology of diphtheria.

Botulinus toxin, as Marinesco (106) and Kempner and Pollack (107) have shown, has a direct action upon the anterior horn cells of the spinal cord with degenerative changes in the Nissl granules.

In filtrates of cultures of *Cl. tetani* two kinds of exotoxins are demonstrable. One of these, referred to as tetanolysin, belongs to the large group of bacterial hemolysins; the other, termed tetanospasmin, has a marked selective effect upon the cells of nervous tissue, particularly those involved in motor functions. The early experiments of Marie and Morax and Meyer and Ransom seemed to prove that unlike many other poisons tetanus toxin was not distributed by the blood stream to the point of final attack but was absorbed from the blood and tissues by the peripheral nerve endings alone and was thence transported centrip-

etally along the motor nerves. Recently Abel (108) has challenged this concept which has long been universally accepted.* On the basis of new experimental evidence he concludes that the toxin is carried to points distant from the site of its origin or injection by the blood or lymph and not by the nerves. He also believes that the toxin has a local action either on the neuro-muscular junction or directly on the muscles themselves, which would account for the symptoms of so-called local tetanus. A note by Zuger and Friedemann (109) in 1938, however, would appear to cast some doubt upon Abel's conclusion. These authors find that many times more tetanus antitoxin given intravenously is required to protect guinea pigs against a given dose of toxin administered intramuscularly than against the same dose when injected intravenously. They also found that free antitoxin was present in the blood of the intramuscularly injected animal at the time of death. These findings, if substantiated, strongly suggest that the death of the animal cannot be due to toxin reaching other muscles or the central nervous system by way of the circulating blood. As the problem now stands, it is obvious that one cannot reach a final decision in respect to the exact route by which tetanospasmin reaches the central nervous system.

Filtrates obtained from certain cultures of staphylococci exhibit toxic properties which are manifested (1) by a hemolytic effect on the red blood cells of certain animals (rabbits and sheep); (2) by the capacity to cause severe inflammation in the skin of rabbits (dermonecrotic reaction); (3) by an immediate lethal action when injected intravenously into rabbits; (4) by an irritating effect on the gastro-intestinal tract of man following ingestion; and (5) by a destructive action on white blood cells, particularly the polymorphonuclear leucocytes. The last two properties are associated with substances distinct from each other and from those capable of inducing the first three types of reaction. Burnet (110) believes that the hemolytic, dermonecrotic, and lethal properties were dependent upon a single toxin. Others, such as Weld and Gunther (111), regard these effects as due to two and possibly three different toxins, each distinguished by its own selective effect on tissues. However this may be in respect to the lethal and dermonecrotic aspects of the poison, it does appear certain from the work of Glenny and Stevens (112) that some staphylococcus filtrates contain two antigenically different kinds of hemolysins (α and β lysins). The gastro-enteric toxin or "enterotoxin" cannot be considered as a true exotoxin, since there is no reliable indication that it induces the formation of antitoxin. This characteristic, together with its greater thermostability, serves to distinguish it from the other staphylococcus toxin or toxins (113).

* Further details of Abel's work are presented in the chapter on Immunity in Tetanus.

Various studies on the lethal effect of staphylococcus exotoxin in rabbits have strongly suggested a selective action on the heart muscle. The latest of these is that of Dingle and his collaborators (25), who summarize the earlier literature and conclude on the basis of this and their own findings that death is caused by direct toxic action on the myocardium. Similarly, Rotberger (114) as early as 1905 attributed the rapid death of rabbits and other laboratory animals after intravenous injection of poisonous filtrates from cultures of Vibrio Nasik (a cholera-like organism) to immediate cardiac injury.

The mechanisms underlying such selective action of these poisons are unknown, although this type of behavior is obviously analogous to the specific action of alkaloids, narcotics, and other drugs. In order that the poison may act upon a cell we must, of course, assume that it has either chemical or physical affinity for this cell. The problem is not unlike that of tissue staining. A dye must be able to form a chemical union with the cell or it must be soluble in the cell substance in order to stain it. The study of drugs of known constitution has revealed relations of this kind which furnish analogies to the selective behavior of bacterial and other complex toxins of biological origin. Consider, for instance, curare, which contains two basic groups, the one a quaternary or ammonium base, and the other a tertiary base, consisting of three methyl groups linked to nitrogen. The latter is only slightly toxic, but by the addition of a methyl group may be converted into the beta methyl base which possesses the typical activity of curare, the so-called curarin. From these and other examples, it is clear that in a certain number of cases actual chemical affinity must play a part in toxic action. On the other hand, there are many instances of poisoning which may depend merely upon physical conditions such as solubilities. Meyer and Overton's well-known theory of narcosis maintains that certain narcotics exert their action by passing out of solution in the blood and lymph into solution in the fat-like substances (lecithin, cholesterin, cephalin) contained in nervous tissue.*

As regards bacterial poisons the union between toxin and susceptible cell is usually firm. This points to the possibility that chemical union takes place rather than merely a loose combination like that of the solution of one substance in another. Furthermore, the complete inactivation of some poisons by mixture with the cells of tissues capable of binding them would likewise point to more than simple physical adsorption, although the possibility of this latter mechanism is by no means excluded by these considerations. Evidence for the relationship between cell union and the activity toxins has been obtained in various ways.

* For Overton's theory of osmosis see Höber (115). Consult P. Th. Müller (116) for a more comprehensive discussion of this entire question.

Dönitz (117) has shown that tetanus toxin injected into the blood stream of a susceptible animal rapidly diminishes in quantity, and Knorr (118, 119, 120), in similar experiments, found that the disappearance of the toxin from the blood is synchronous with the appearance of symptoms, a fact which excludes disappearance by excretion. Conversely, Asakawa (121, 122) pointed out that in pigeons, which are but slightly susceptible to tetanus toxin, the poison could be detected in blood, liver, spleen, kidneys, and muscles six days after injection, but not in the brain. Similar to these results are those of Metchnikoff (123), who found this poison unchanged after two months in the circulation of insusceptible animals such as the lizard. Direct evidence of union between susceptible tissues and poison has been furnished by the experiments of Wassermann and Takaki (124), who showed that the brain and cord of rabbits and guinea pigs, mixed with tetanus toxin before injection, served to neutralize its harmful effects. And it appears that this property of nervous tissue to neutralize the poison is proportional to the susceptibility of the species to its action. Thus Metchnikoff (125) not only confirmed the findings of Wassermann and Takaki in respect to rabbits and guinea pigs, but also showed that the brains of chickens — animals that are but moderately susceptible — possess a correspondingly slighter neutralizing power, whereas those of completely resistant cold-blooded species, such as turtles and frogs, have no inhibiting power whatsoever.

Similarly, it can be demonstrated that hemotoxins and leucocidins are bound by those cells which are specifically vulnerable (111, 126).

A number of attempts have been made to determine chemically the cell constituent which united with the poisons. Noguchi (127) showed that cholesterin and alcoholic extracts of blood serum neutralized tetanolysin. The same thing was later shown by Müller (128), and Landsteiner (129) showed that ether extracts of red blood cells likewise neutralized this poison. In a later study by Landsteiner and von Eisler (130) they found that extraction of blood cells with ether rendered the stromata less capable of binding the hemolytic substances. Landsteiner and Botteri (131) also worked with tetanospasmin and found that protagon obtained by alcoholic extraction of human brain possessed a powerfully neutralizing effect upon the toxin, greater than that possessed by other lipoidal substances; 0.15 gram neutralized 120 minimal lethal doses of the toxin. Takaki (132) isolated an alcohol-soluble element, cerebron, from nerve tissues, to which he ascribes the toxin-binding properties. Overton and Bang (133) found that cholesterin and lecithin inhibit the action of cobra venom, a poison which is in so many ways similar to those produced by bacteria. Taking into consideration all available evidence, it seems that the lipoids play an important role in determining the selection of the nervous system by the bacterial poisons.

it may not, of course, be an influence depending merely upon the solubility of the harmful substances in the lipoids themselves. For, as Bang expresses it, "the lipoids possess to a high degree the property of altering by their presence the solubilities of other bodies," and it is quite possible that in the tissues they are present as lipoid-protein combinations. It is of some interest in this connection to recall the experiments of De Waele (134), which bring out another clear analogy between alkaloids and bacterial poisons in their relation to lecithin. He found that the addition of small quantities of lecithin increases the activity of both toxins and alkaloids in the animal body, whereas larger amounts inhibit both.

The extreme toxicity of certain of the recently purified exotoxins of *Cl. tetani* and *C. diphtheriae* lead to speculation upon the manner in which they may injure the cell, inasmuch as it may be calculated on the basis of the M.L.D. and the probable molecular weight that only a relatively few molecules of toxin must be sufficient to damage or destroy a single cell. This suggests that the toxin may inhibit some essential enzymatic process or coenzyme system in cellular metabolism, in much the same way as minute traces of certain chemical substances such as mercury inactivates urease, and cyanide certain oxidation-reduction systems.

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CHAPTER IV

THE BASIS OF IMMUNITY

In our preliminary discussion, we have emphasized the fact that every infectious disease is the result of a struggle between two variables — the pathogenic powers of the bacteria on the one hand, and the resistance of the subject on the other — each of these again modified by variations in the conditions under which the struggle takes place. Thus a microorganism may be capable of causing fatal infection in one individual but may be only moderately virulent or even entirely innocuous for another. Conversely, the same individual may be highly susceptible to one variety of bacteria but highly resistant to others. Even in reactions with one and the same microorganism, the susceptibility or resistance of the individual may be determined by variations in the physiological state or by the environmental conditions under which the two factors, invader and invaded, are brought together. Therefore, the conceptions "resistance," "immunity," and "susceptibility" are relative terms which can never be properly discussed without consideration of all modifying conditions.

The science of immunity deals with a detailed analysis of these variables. Its practical aim is the determination of methods by which susceptibility can be transformed into resistance.

It has long been recognized that there are various kinds of immunity. Universal experience, for example, teaches us that one attack of measles usually confers lifelong resistance upon an individual, or that such animals as the cat and dog, although they may be in intimate contact with a patient during the course of that disease, never themselves contract an infection of the same nature. Here we have obviously two types of resistance — one which is conferred by an attack of the malady upon a susceptible host, the other apparently associated with a natural refractory state exhibited by all members of a species. On the basis of epidemiological and experimental data, immunologists have attempted to classify the different varieties of resistance which have been observed to occur naturally or which develop as a result of artificial immunizations. It is possible to think of immunity to a given infectious agent as con-

ditioned by innate factors which are transmitted according to the laws of heredity from one individual or generation to the next and which consist in physiological mechanisms entirely independent for their appearance or functioning of stimuli arising from contact with the environment. There is, as we shall see, ground for believing that this sort of immunity does exist. On the other hand, an increased resistance may follow as a result of the particular environmental conditions encountered either by an individual or a group of individuals belonging to a species which is more or less susceptible to infection by a given pathogenic agent. We have then two broad divisions of immunity: that which is innate and that which is acquired. It must be made clear at once that in many instances it has been impossible to decide whether or not the resistance of a whole species against a given disease is due to inherited factors or to universal contact with the causative organism or another possessing a similar or identical antigenic constituent. Indeed, only a very few unequivocal examples of immunity attributable solely to mechanisms genetically transmitted can be cited, and these are to be found in differences exhibited by strains or varieties within the species. Our meager knowledge of inherited immunity will be presently summarized.

Two other large categories have been established according to the criteria of whether the resistance is naturally present or whether it appears only after manifest infection or artificial immunization.

Unquestionably, the distinction between natural and acquired immunity without further qualification, although convenient, is purely formal, since it is known that the natural immunity of an individual or group may depend upon inapparent infections or contacts with the infectious agent which, while causing no manifest disease, yet measurably decreases the susceptibility of the host.

It would then be clearer as well as more accurate to regard, as Topley (1) is inclined to do, every type of increased resistance which does not arise genetically as acquired. Such immunity may, however, be acquired naturally as the result of either open or occult infection or the transmission of antibody from mother to offspring *in utero*, or, on the other hand, artificially, following the administration of vaccines or serum containing specific antibodies.

When immunity follows infection or vaccination with materials containing the pathogenic agent or its products in some form, it is characterized as "active immunity" in contrast to the "passive immunity" which is conferred by the transfer of antibody whether naturally from the mother via the placenta, or artificially by the injection of antiserum derived from an actively immunized animal or individual.

We may summarize what has been said concerning the various types of immunity in the form of a table.

1. Innate Immunity — Natural
2. Acquired Immunity
   - *a.* Natural
     - Active
     - Passive
   - *b.* Artificial
     - Active
     - Passive

There can be little doubt that all these various types of immunity may occur within individuals, races, and species, although in our present state of ignorance it is frequently difficult or impossible to distinguish between innate immunity and natural immunity acquired as the result of inapparent infections.

Natural Immunity

The Inheritance of Immunity — Racial Resistance. The problem of the role of inherited factors in immunity has only recently been the subject of intensive experimental investigation by means of adequate techniques, although a few observations of earlier works such as those of Müller (2) on the difference between the resistance of white and black rats and the limitation of susceptibility to the Rous chicken sarcoma to Barred Plymouth Rocks and a few other races of chickens, as well as the well-authenticated fact of the greater resistance of Algerian sheep to anthrax compared with those of Europe, clearly indicated that a correlation existed between breeding and resistance to infection. The pertinent literature up to 1934 has been summarized and carefully evaluated by Hill (3). Most of the work has been done either with chickens or mice and rats as the experimental animals, although guinea pigs and rabbits have in a few cases been employed. According to Hill, the results derived from the studies on chickens, with the possible exception of those of Lambert (4, 5), fail to yield any conclusive answer to the question of whether genetic factors can account for the differences noted in resistance to bacterial infection. Lambert's experiments, while revealing no valid evidence for genetic differences in resistance to infection with *B. Gallinarum* between three breeds (White Plymouth Rock, White Wyandotte, and Rhode Island Red), strongly suggest that selective breeding within the breed produced individuals which are more resistant to this particular pathogen than unselected stock. Although the opportunity for the appearance of either natural active or passive immunization cannot be entirely excluded, these alone are unable to account for the observed increase in immunity. Tyzzer (6) and Gowen and Schott (7) have studied the differences in resistance exhibited by various breeds of mice to a peculiar organism called by Tyzzer *B. piliformis*. Whereas Japanese waltzing mice proved highly susceptible to this pathogen, the other strains of mice tested proved relatively insuscep-

tible. In Gowen and Schott's experiments, among the waltzing mice 80 per cent were found to be susceptible in contrast to the complete resistance shown by a strain of silver brown mice. When crossbred, the progeny of these two breeds in the F_1 generation were all resistant in accordance with the tentative hypothesis that a single major factor accounting for resistance in the silver brown and susceptibility in the waltzing mice was involved. Back crossing yielded further evidence in support of this conception of the heritable mechanism.

The most extensive researches on innate immunity have been carried out by Webster and his associates at the Rockefeller Institute. It is impossible here to describe adequately the large amount of experimental results which they have presented, and the reader is referred to the reviews by Webster (8), Hill (3), and Greenwood and his associates (9) for bibliography and critical analyses of the data. In brief, however, Webster (10), by selectively breeding from the Institute strain of white mice, obtained a number of lines which, when tested by feeding *B. enteritidis*, proved either significantly more susceptible or more resistant than the unselected stock mice which were used as controls. Among the lines bred for susceptibility, the mortality rates of some were very high (70–90 per cent) compared with the average rate for the controls (about 37 per cent). In other lines, however, the mortality rate failed to rise to this degree and the slight increases noted may be of doubtful significance. Since elaborate precautions were taken to rule out the factor of acquired resistance and fluctuations in virulence of the infecting organism, the results involving large numbers of animals in all probability indicate that susceptibility to *B. enteritidis* can be increased by selection. Evidence for the development of resistant stock through the use of the same method is even more convincing. A number of lines were bred which revealed mortality rates less than half that of seventeen groups of control stock mice. Thus two lines developed in the course of the fourth selection which had mortality rates of 17.8 per cent and 15.5 per cent, respectively, compared with the average rate of 37.4 per cent for the controls, although the former received ten times the infecting dose of organisms. Further support for the validity of these findings was obtained by means of cross-breeding experiments and the exposure of selected lines of mice to herd infection. Further work (11) on the relative susceptibility of various special strains of mice showed that they differ very significantly in their mortality rates when infected with *B. enteritidis*. Comparing these results with those Webster obtained by infecting certain of these strains of mice with three other bacterial species (*Past. Avicida*, Friedländer's Bacillus, and Pneumococcus) and the virus of louping ill, Hill finds that there is indication of a considerable degree of specificity in the activity of the genetic factors for resistance or suscepti-

bility, since three strains equal in their susceptibility to *B. enteritidis* proved unequal in their behavior to infection with these other pathogens. Of great interest is the observation that the Rockefeller strain of mice resistant to *B. enteritidis* were highly susceptible to the virus. Webster (12) in his earlier work had asserted that susceptibility and resistance were probably entirely non-specific, since he found parallel responses among selected mice to such widely different harmful agents as *B. aertrycke* and mercuric chloride.

Similar investigations on mice by Pritchett (13, 14) and by Gowen and Schott (15, 16, 17) support those of Webster. With the same objective in view, Irwin (18) has carried out breeding experiments with rats which were then tested with the Danysz bacillus. Significant differences in the mortality rates appeared in the progeny of resistant parents. In his critique of the results, Hill concludes that the factor of acquired immunity cannot account entirely for the findings, although it is impossible to exclude it completely as a possible adjuvant in increasing the resistance of the selectively bred animals. Experiments of Topley and Wilson (3), which have been summarized by Hill indicate how important this factor may be in experiments which involve breeding from parents which have survived infection.

On the basis of this experimental evidence, we may, however, conclude that differences in resistance to a number of infections in certain animals can be in part attributed to variations in the genetic pattern and that such differences certainly occur between strains as well as most probably between individuals within the strain.

As yet, practically nothing is known of the immunological basis for inherited immunity of this sort. Before more than a beginning can be made in the analyses of the physiological factors concerned, the important question as to specificity must be definitely answered, since if innate immunity was found to be more or less directed against particular varieties or groups of infectious agents, it would be profitable to investigate the possibility that the presence or absence of specific antibodies might be responsible. In their studies on the inheritance of immunity in rats, Irwin and Hughes (19, 19 a) were able to obtain a certain degree of correlation between resistance and the presence of bactericidal power in the whole blood, since, when an animal's blood failed to kill any bacteria, the animal died; while about one half of the animals which yielded bactericidal blood survived. When such observations as these are supplemented with many others of the same sort and the specificity of the bactericidal action is analyzed, we possibly may be in a better position to describe the mechanism upon which innate immunity depends.

We have dealt at some length with the experimental aspects of inherited resistance, since here seems to lie the most trustworthy evidence

which we possess at present for regarding it as a real phenomenon. In man, the many reported differences between races or groups are much more difficult to analyze. Indeed, most of these can usually be traced to factors that do not constitute true racial immunity, such as diet, poor living conditions, isolation from contact with certain agents of disease, social and religious habits — all of which are hard to appraise. For example, it has frequently been remarked that the greater susceptibility of the Negro in the United States compared with that of the white races to tuberculosis may depend upon genetic factors peculiar to the former race. But one can urge with equal cogency that this well-established fact may be attributed to adverse environmental conditions or that both sets of factors are concerned (20, 21, 22, 23, 24, 25). In this connection, the results of the recent survey carried out by Kahn (26) among the Bush Negroes of Dutch Guiana are of interest, since they show that although opportunity for infection is present, this is very infrequent, as revealed by the extremely low rate of positive tuberculin tests (2.34 per cent), and the great rarity of clinically recognizable disease. The isolation of the group which have lived under the natural conditions to which they were accustomed in Africa before transportation as slaves to America may account in part for these unusual findings. Kahn, however, suggests that the favorable circumstances of their life may be of considerable importance in bringing about this unusual situation.

The reputed racial resistance of Negroes to yellow fever is without much doubt due to mild childhood disease. This point of view has been maintained in the past by Hahn (27) and others and is confirmed by the recent epidemiological studies summarized by Sawyer (28) and carried out by members of the International Health Division of the Rockefeller Foundation in areas where yellow fever is endemic, using the mouse protection test as an index of previous infection. Similarly, the resistance of Negroes to malaria and of Mexicans to smallpox and typhus fever are likewise in all probability due to unrecorded attacks of the disease sustained at an early age.

On the other hand, it is possible that the extraordinary susceptibility of various aboriginal peoples to such newly introduced diseases as measles, syphilis, and tuberculosis may be due to the fact that no opportunity has arisen for the natural selection of more resistant individuals to take place through the weeding out of the most susceptible by death from the disease. Thus various authors have surmised that the constant decline in morbidity and mortality rates of tuberculosis in the United States and elsewhere that has obtained during the last fifty years or more may be partly conditioned by selection of this sort with the emergence of a genetically more resistant stock. It has been suggested that the decline of leprosy in Europe from its high incidence in the Middle Ages to its

almost complete disappearance at the present time may be due to the operation of the same process. Certainly this disease exhibits a marked tendency to appear in a number of individuals of the same family. Hopkins (29) has studied the occurrence of leprosy from the familial point of view and concludes that his data suggest a hereditary lack of resistance. The fact that he found only a small number of cases in husband and wife, whereas blood relatives revealed a high incidence in leprous families, supports his findings. Hopkins' analyses of the cases of leprosy at the National Leprosarium at Carville, Louisiana, also point to a racial susceptibility of Mexicans compared with Americans of European origin, whereas the Negroes of Louisiana would seem to be more resistant than either of these other races.

Surveys carried out by means of the Wassermann and Kahn tests by Shattuck (30, 31, 32) and his associates among the Maya Indians of Yucatan and Guatemala show that although infection with syphilis is present, the clinical manifestations of the disease even in the absence of modern methods of treatment are usually extremely mild. Shattuck raises the question as to whether this enhanced resistance may not be attributed to a long process of selection.

It has been rather generally held that the white races are refractory to yaws, at least to the disease in its typical form. Cases of infection have, however, been reported in whites, although they are admittedly rare. Since there is a very close relationship between yaws and syphilis and considerable evidence which points to a cross-immunization effect, it is difficult to be entirely certain concerning the greater susceptibility of dark-skinned races, although this seems quite probable.

From the foregoing considerations, it is manifest that racial immunity among human beings is certainly attributable to acquired resistance in most instances. But the possibility exists none the less that genetic factors may be occasionally involved. Moreover, it is extremely difficult even under the conditions of animal experimentation to determine in any given case which type of immunity is responsible for the observed results. Still more uncertain is the nature of the mechanism underlying innate resistance.

Species Differences in Immunity. Although here too the separation of environmental factors, particularly that of active immunization by inapparent infections, is difficult, it is logical to think that the remarkable resistance of certain species against pathogens which are highly virulent for others is frequently due to constant inherited factors which breed true. We may convey the clearest conception of all such species differences by a tabulation of some of the more important diseases of man, together with the relative resistance of various other animal species to these same infections.

Tuberculosis, human type, spontaneously infects man. It is very often observed in monkeys kept in captivity. Cattle, swine, and sheep are probably never spontaneously infected; guinea pigs are highly susceptible to experimental inoculation. Cattle, swine, sheep, and rabbits are relatively resistant to experimental infection. Dogs and goats are still more so. Birds seem to be entirely refractory.

Tuberculosis, bovine type, spontaneously infects domestic animals, chiefly cattle. It is less frequent in sheep, hogs, and horses; and it has been reported in dogs and goats. In man, infection *does* occur, but only a relatively small percentage of human tuberculosis is of the bovine type, and these cases are almost exclusively in children.

Experimental infection is successful in rabbits and guinea pigs, both of these animals succumbing more rapidly to this than to the human bacillus. In fact, the relative resistance of rabbits to the human bacillus is such that rabbit inoculation is one of the most important methods of differentiating between the two types. Birds are refractory.

Tuberculosis, avian type, occurs spontaneously in birds. It may be experimentally produced in rabbits (Straus and Gamaleia). Injected into cattle, it causes a local reaction only. It may rarely cause disease in man.

Tuberculosis of cold-blooded animals is not transferable to warm-blooded animals.

Syphilis spontaneously occurs in man only. It can be inoculated into chimpanzees, in which primary and secondary lesions develop, corresponding to mild human syphilis. Primary lesions can be produced in lower monkeys. It can be transferred by intratesticular inoculations to rabbits.

Gonococcus infection occurs spontaneously in man only. No typical lesions can be produced in experimentally inoculated animals, though death can be caused by large doses, probably by toxic action or through the simultaneous inoculation of mucin.

Influenza bacillus spontaneously infects man only. Experimental infection of the meninges of monkeys can be accomplished when large doses are employed. With the aid of mucin, mice may be infected and killed by comparatively small doses.

Glanders occurs spontaneously in horses and mules; less frequently in sheep, goats, and camels. This disease, like plague, may be regarded as primarily a disease of animals, but man may be infected by direct or indirect contact with the diseased animal. All domestic animals may be infected experimentally with ease, except cattle and rats, in which cases large doses are necessary. Birds show local reactions only.

Plague occurs spontaneously chiefly in man and in rats. The tarbagan is also a natural host. It has likewise been found in California ground squirrels and in hogs during plague epidemics in Hong Kong. It is highly infectious for guinea pigs and white rats, slightly less so for mice; rabbits are much less susceptible than guinea pigs. Dogs, cats, and cattle are relatively resistant. Birds appear to be immune. Cold-blooded animals are immune unless artificially warmed.

Malta fever occurs spontaneously in man and in goats. The causative agent, *B. melitensis*, is pathogenic for all mammals, but it is not fatal for lower animals when the organisms are directly cultivated out of the human body. Closely related is the disease caused in cattle by the *Bacillus abortus bovis*, which can also infect man. We believe that it is not unlikely

that *B. abortus bovis* is a mutant of *B. melitensis* through passage in cattle, analogous in relationship to it as is bacillus of bovine tuberculosis to the human type.

Diphtheria occurs spontaneously in man only. Experimental inoculation is fatal in guinea pigs, rabbits, dogs, cats, and birds. Rats and mice are highly resistant. The typical pseudomembranous inflammation can be produced in susceptible animals only after previous injury of the mucous membrane, and then it rarely shows any tendency to spread.

Tetanus is spontaneous in man, horses, cattle, and sheep. It is found rarely in dogs and goats. Birds are highly resistant to experimental inoculation.

Anthrax is primarily a spontaneous infection of cattle, sheep, and horses; it occurs in man largely through direct or indirect contact with these animals. Guinea pigs, rabbits, and white mice are very susceptible to experimental inoculation. Rats and hogs are less susceptible, and dogs are relatively resistant, though they can be regularly killed by moderate doses intravenously injected. Birds and cold-blooded animals are highly resistant.

Asiatic cholera develops spontaneously in man only. Rabbits and guinea pigs can be killed by injections of cultures, but die probably of toxemia. Ordinarily, no multiplication takes place in the animal body. Pigeons are insusceptible, a fact which helps to distinguish this organism from *Spirillum metchnikovi* and other similar bird-pathogenic spirilla.

Typhoid fever occurs spontaneously in man only. It has been produced in a mild form in chimpanzees. Animals are susceptible to the endotoxins and can therefore be killed by injections of bacilli and extracts, but the organism is not invasive as in the case of the lower animals. Typhoid septicemia can be produced in rabbits and mice by inoculating them with especially virulent cultures of the bacilli, or cultures previously grown on rabbit-blood agar. The typhoid-carrier state may ensue for considerable periods in such animals.

Pneumococcus infection in various forms occurs spontaneously in man and guinea pigs. Rabbits and mice are highly susceptible but usually fail to develop a pneumonia, since they rapidly succumb with a generalized invasive of the blood stream. Rats, dogs, cats, cattle, and sheep are relatively resistant.

Leprosy occurs spontaneously in man only. Lesions simulating human leprosy have been produced in monkeys by inoculation, and partially successful experiments have been made upon the Japanese dancing mouse. Other animals are immune.

Staphylococcus and streptococcus infections may occur in almost all of the warm-blooded animals.

Scarlet fever, a disease caused by the streptococcus, occurs spontaneously in man only. Monkeys may possibly be susceptible, though not all observers have been successful in such experiments. The erythrogenic toxin of *Str. hemolyticus* causes rashes in man and skin lesions in rabbits.

Smallpox occurs spontaneously in man only. The same virus, altered by animal passage, causes cowpox in cattle. It can be experimentally produced in monkeys in a modified form.

Measles develops spontaneously only in man. *Macacus rhesus* has been successfully inoculated by Anderson and Goldberger, and Blake and Trask. Other animals are immune.

Typhus fever occurs in man only. Experimentally it has been produced in chimpanzees, *Macacus*, *Cercopithecus*, *Ateles*, and *Mycetes* monkeys. Nicolle was the first to show that in guinea pigs this virus will produce a characteristic temperature curve without death. It is not impossible that in this disease as well as in Trench Fever we may be dealing with a condition that is primarily a disease of insects (lice) transmissible to man. It also occurs as an inapparent infection in rats.

Yellow fever is transmitted to man by the *Aëdes egypti* mosquito and can be experimentally produced in *Macacus* monkeys and, in a modified form, by intracerebral inoculation in mice.

Poliomyelitis is spontaneous in man only. It can be transmitted to monkeys. No other animals are known to be susceptible.

The above represents an incomplete tabulation of the variations in susceptibility in the animal kingdom for infections which occur spontaneously in man. We might with equal profit tabulate the infections occurring spontaneously in any single species of animal, and show how variable would be their pathogenic powers for other animals and for man. Thus man is immune to the organism which causes cattle plague, and to that of chicken cholera, and probably to many other diseases peculiar to animals, though of course in the case of infections of the human being we are entirely dependent for such information upon observed immunity to spontaneous infection and upon a few instances of accidental inoculation.

A survey of the data included in the table also reveals a fact of great importance in the study of immunity in general. In many instances, the immunity of even a resistant species is only relative. By taking measures to reduce the resistance or by giving massive doses of the infectious agent, disease and death not infrequently ensue. Instances of absolute immunity of species are relatively rare. The most striking examples within the mammalian group are perhaps the absolute resistance of man to cattle plague and that of the lower animals, with the exception of the monkeys, to measles. Among races and individuals belonging to a susceptible species, probably none are so naturally refractory that adequate dosage or depression of the resistance through unfavorable environmental conditions will not serve to inaugurate infection, although the immunity resulting from an attack of some diseases — particularly those due to filterable virus agents — appears usually to be of a very high order.

There is no single factor upon which we can say that the resistance of a species against a particular organism or virus depends. In a few instances, we find a possible mechanism in the unfavorable environmental conditions offered to the bacteria within the host. Thus, as we have seen in Chapter I, the fact that some diseases of warm-blooded animals do not affect the cold-blooded species and vice versa is without doubt a

matter of temperature. This is clearly revealed by the classical experiments of Pasteur, Joubert, and Chamberland (33), who were able so to decrease the resistance of chickens to anthrax bacillus by lowering the body temperature that the bacteria were able to invade and kill these normally refractory animals. Conversely, Gibier (34) infected frogs with anthrax by keeping them in water at 35° C., and Nuttall (35) secured a similar result with plague bacilli in lizards by holding the latter at a temperature of 26° C.

Differences in metabolic processes between species by affording favorable or unfavorable growth conditions may likewise present barriers against infection. Thus, variations in the normal bacterial flora of the gastro-intestinal tract, in the amount and kind of enzymes produced therein, as well as in the hydrogen ion concentration, both of secretions and the blood itself, might afford unsuitable milieus for growth.* Long (36) has conceived of a possible relationship between the degree of oxygen tension, carbon dioxide production, water relations, and the availability of free glycerol in the tissues and the ability of the tubercle bacillus to survive and multiply. Such hypotheses, although logical enough, have at present little or no direct experimental support which would allow us to consider them as representing more than interesting ideas and points of departure for further investigation. Much more definite, in certain cases at least, is the role of natural antibodies and phagocytic cells in species immunity. We will, therefore, briefly outline here the researches which demonstrated parallelism between these factors and resistance of species as a whole, although it should be borne in mind that they are also of fundamental significance in the immunities of groups and individuals belonging to a susceptible species.

The early investigations upon natural resistance as distinguished from artificial immunity were stimulated by Pasteur's success in protecting susceptible animals against a variety of infections by active immunization with various types of vaccines which will be subsequently described. Since it was soon determined that there were no gross alterations in anatomical structure or functional activities which could account for the resistance thus artificially induced, it seemed to those who first attempted to analyze the changes following active immunization that a clue might be obtained through the study of the reactions in naturally resistant and naturally susceptible animals. It was hoped that natural resistance might be found to depend upon some readily recognizable behavior of the body fluids or cells which would explain the physiology of immunity.

Two lines of investigation were begun almost simultaneously, one centering upon the activities of cells especially as manifest in local

* See Chapter I.

inflammations, the other upon the possible protective powers of the body fluids, particularly the blood plasma.

In 1874, Panum had called attention to the presence of microorganisms within white blood cells and suggested that the organisms were destroyed by intracellular digestion. Metchnikoff in 1883, before the Naturalists Congress at Odessa, ascribed the absorption of dead corpuscular elements in the bodies of invertebrates to the action of certain phagocytic cells. Later he studied the infection of the Daphnia or water flea (a small crustacean) with yeast cells and determined that recovery or death of the Daphnia depended upon the degree to which the invading yeast was taken up by the phagocytic cells in the body cavity. Subsequent efforts of Metchnikoff and his pupils to trace parallelism between natural immunity and phagocytic power were successful in many cases but in others no such parallelism appeared to exist.

Others turned to the blood plasma as possibly responsible for the protective mechanism, largely because of observations like those of John Hunter, of Traube and Gscheidlen (37), and of Lord Lister (38), who had noted that shed blood did not putrefy as rapidly as did many other organic substances. In 1884, Grohmann (39) determined the inhibitory action of cell-free blood plasma upon bacteria; Fodor (40), in 1887, and Nuttall (41), in 1888, showed that fresh normal blood possessed the power of actually killing bacteria (bactericidal power). Nuttall repeated Metchnikoff's (42) experiments on anthrax, in which he had shown the phagocytic destruction of these organisms in rabbits, and confirmed the observations, but interpreted them as indicating that the phagocytosis merely removed the bacteria killed by the blood plasma. Nuttall detected similar bactericidal properties in pleural exudates, pericardial fluids, and aqueous humor, and determined that this property was "inactivated" or destroyed when the fluids were heated to 55° C. for ten minutes or longer. Buchner (43) confirmed Nuttall's results and showed further that the bactericidal property resided, not only in defibrinated blood, peptone blood, and plasma, but was present also in the serum obtained after clotting. He applied the term "*alexin*" to this active constituent of the blood — likening its action to that of a ferment. To this substance — the heat unstable, active constituent of fresh blood — Ehrlich later applied the name "complement."

The result of these discoveries was an attempt, by Flügge's school, to base natural as well as acquired resistance solely upon the bactericidal properties of the body fluids. The observations of Nuttall and Buchner were soon extended to peritoneal and other exudates by Stern (44), and to ascitic fluids by Prudden (45).

In respect to the relationship of the bactericidal effects of body fluids and natural immunity, it was shown by Nuttall, Buchner, Nissen (46),

and their immediate followers that the blood serum of many resistant animals possessed bactericidal properties against many microorganisms. However, just as in Metchnikoff's studies (47), a constant correlation between bactericidal power and the degree of natural resistance could not be established in every instance.

The reconciliation between these opposing "humoral" and "cellular" schools came about through the work of Denys, Gruber, and Futaki, of Wright, Neufeld, and others, who showed that certain serum constituents unite with bacteria which thus were rendered susceptible to ingestion by phagocytes. In this way a bridge was established between the two opposing views, although it is now fully recognized that humoral factors alone may be sufficient to destroy certain species of organisms. Whether or not phagocytic cells without the aid of serum can digest bacteria is still open to question, although there can be no doubt that ingestion may sometimes occur under these circumstances.*

In general it may be stated that the results of the pioneer workers as well as many that have since been reported, such, for example, as those of Robertson and his colleagues (48), have made it quite clear that in numerous species the resistance to a particular pathogen such as the pneumococcus is frequently associated with the capacity of the cells and fluids to exert upon it an inhibitory or destructive action. But it should be borne in mind that this correlation may not always be present.

Species immunity, however, cannot be explained by any single mechanism. In some cases one of the factors which has been mentioned may be of especial significance, but we should avoid the conclusion that it is the only one involved. Others which we are as yet unable to recognize may well be operative.

As we remarked at the beginning of this section, species immunity is *a priori* most easily understood by assuming that it is dependent upon constant inheritable factors, but except in those relatively few instances where there is reason to believe that the normal physiological processes of the species offer an unfavorable site for the development of the infectious agent, we cannot be sure that environmental contacts do not lead to increased resistance either through active immunization or through non-specific factors of some sort. In those cases where species immunity appears to be correlated with the presence of natural antibacterial properties of the body fluids, we cannot say whether or not they have developed through contact with the pathogen or with one possessing similar antigenic components,† or whether like the antibodies

* For references and further details concerning this problem the reader is referred to Chapter XI.

† For a thorough review of the evidence which shows that bacteria and other antigenic substances derived from widely diverse sources may share antigenically similar constituents, see Ingalls (49). A number of examples will also be found in Chapter II.

concerned in the human blood groups they arise as the result of a genetic pattern. Until animals are reared in an environment free of bacteria, it would seem that no conclusive answer to this question will be found.

Individual Differences of Resistance in the Same Species and Race. Inasmuch as the individual is a member of its species and race, it will in general exhibit immunological behavior characteristic of these groups to which it belongs. But since variation is a fundamental biological principle, any given individual will depart to a greater or less degree from the curve representing the average resistance or susceptibility of strain or species. Again such individual variations will be conditioned presumably by inheritance and most definitely by environmental experience.

Among species of animals readily susceptible to certain infections, resistance, or immunity, may be acquired by an attack of the disease. Thus human beings who have recovered from plague, smallpox, typhoid fever, cholera, the exanthemata, mumps, typhus, yellow fever, and a number of other conditions do not ordinarily contract the disease a second time. In some of these conditions, notably cholera, plague, typhoid fever, and smallpox, the rule is almost invariable. In others, such as measles, scarlet fever, and mumps, a second attack may occur, though it is rare. A few infections like pneumonia and influenza may recur at relatively short intervals.*

The following table briefly indicates some of the infectious diseases in which permanent immunity follows an attack:

Infectious Diseases in Which One Attack Conveys Lasting Immunity

Plague.
Typhoid — second attack rare.
Cholera.
Smallpox — second attack very rare.
Chicken-pox — second attack very rare.
Scarlet fever — second attack about 0.7 per cent reinfections.
Measles — second attack uncommon, but less rare than scarlatina.
Yellow fever.
Typhus fever.
Mumps — second attack rare.
Poliomyelitis.

* In appraising the value of our knowledge of permanent immunity after disease, a simple mathematical consideration will indicate that we may have overestimated this security. If, for instance, in a given place 0.2 per cent of the population is likely to have typhoid fever, of the 0.2 per cent who have had the disease, only 0.2 per cent again would be likely to get it were they not immune. If we consider, further, that those who have had it are growing out of the age in which exposure to typhoid is most common, the normal likelihood of a second attack is further diminished. Thus second attacks could be expected in less than 0.2 of 0.2 per cent or in 0.04 per cent. Unless statistics are corrected by these considerations, we really have little knowledge of the degree to which a single attack protects permanently in many of these diseases.

No lasting immunity is conferred by one attack in:

Infection with the Pyogenic cocci
Gonorrhea
Pneumonia
Influenza
Glanders
Dengue fever
Diphtheria in general protection, second attack in 0.9 per cent cases
Recurrent fever
Tetanus
Erysipelas
Tuberculosis, Syphilis } see below

There is another group of diseases in which the immunological conditions after infection are not entirely clear — namely, protozoan infections like trypanosomiasis, and treponema diseases like syphilis. In these conditions reinfection seems to be impossible so long as the individual still harbors the microorganism, but no lasting immunity is conferred.* In tuberculosis, also, resistance to superinfection is dependent on the presence of a focus. Similarly, with some of the virus diseases it has been postulated that immunity may be the result of persistent occult infection, and lasts only so long as the pathogenic agent remains in the body.† The recent studies of Coggeshall and Kumm (51) and Eaton (52) on the malaria of monkeys suggest that in this disease, which is usually included among the maladies in which an "immunity of infection" alone obtains, resistance may depend upon the development of antibodies which can mediate the destruction of the parasites.

A discussion of the most significant role of infections which do not lead to clinically recognizable symptoms — the so-called "inapparent" infections — and the importance of the congenital transfer of antibodies from immune mother to offspring can best be revealed in a compendious account of the correlation between age and susceptibility.

The existence of a relationship between age and susceptibility to infectious disease is demonstrated by a large body of clinical, epidemiological, and laboratory reports.‡

The factors which underlie differences in resistance exhibited by individuals of various age groups are undoubtedly in many cases due to

* Chesney (50) and others in recent years have obtained evidence from experimental syphilis in animals that immunity may possibly persist for some time after the elimination of the treponemata from the body, but not all agree with this conception. See Chapter XVIII.

† See Chapter XVII.

‡ Ssacharoff (53), among others, has summarized many observations on the relation of age to infections both in man and in animals.

naturally acquired immunity which may be either passive or active.* It is well recognized that infants under six months to a year in age are refractory to infectious diseases which they later contract with great readiness. Many of the childhood exanthemata such as measles, scarlet fever, etc., do not usually occur in these very young children, nor is the Schick test (55) which indicates susceptibility to diphtheria, or the Dick test (56) which has the same significance in scarlet fever, positive in a large percentage of the group. This short-lived immunity depends upon the congenital passive transfer of antibody from mother to offspring either through the placenta or by way of the colostrum.

Chauveau (57) was one of the earliest to observe this type of immunization. He showed that lambs thrown by ewes immune to anthrax were more resistant than those thrown by normal ewes. Observations analogous to that of Chauveau have been made on symptomatic anthrax by Thomas (58) and Kitasato (59). Ehrlich (60), who studied the congenital transfer of antibody in mice immunized with ricin and abrin, proved that the resistance of the mother was transmitted to the offspring by means of the antitoxin in both blood and milk. The second generation of the immunized female is not immune. Among the instances of passive congenital transfer in human beings which has been most studied is that of the passage of diphtheria antitoxin from mother to offspring. Von Groer and Kassowitz (61), for example, in testing 143 mothers and their infants by means of the Schick test and by titrations for antitoxin in the blood, found that in nearly all cases mothers who were Schick positive and contained no antitoxin in the blood possessed infants who were likewise Schick positive and antitoxin negative. Conversely, Schick negativity and antitoxin positivity distinguished the mothers and offspring in an equally large percentage of cases (96 per cent).

The problem of the route by which such passive immunization is conferred has been extensively investigated. Apparently contradictory conclusions reached by various workers can be explained on the basis of the differences in the anatomy of the placenta of different species. In pigs and ruminants there are respectively five and four layers of cells between the maternal and foetal circulation. Passive immunity in these animals by the placental route does not appear to take place but is transmitted entirely by the colostrum. In contrast only one layer is interposed between the two circulatory systems in rodents, apes, and man, and passive immunity is predominantly placental in character. Thus Famulener (62) showed that in highly immunized goats, hemolytic antibodies passed to the kid from the milk. Theobald Smith (63) studied scours, a generalized colon bacillus infection in calves, and found

* Baumgartner (54) has reviewed the literature up to 1932 on the relationship of age to immunological response.

that those that succumbed were the ones that have never had colostrum, having been reared without suckling, since in the colostrum antibodies for the bacteria pass from cow to calf. In man, as Kuttner and Ratner (64) and many others have shown, diphtheria antitoxin and other antitoxins pass mainly through the placenta, and not through the milk.*

It is clear, then, that depending upon the immunological state of the mother in respect to specific circulating antibodies, a given infant will for a time be more resistant to certain infections.

Following the six months or longer during which this passive immunity is effective the child loses its antibodies and becomes susceptible. We then have the period of the common childhood diseases, most of which leave in their wake a lasting actively acquired immunity. The immunity of adults to measles, mumps, whooping cough, and chicken pox is due almost if not entirely to obvious attacks in childhood. Thus, it has been shown in isolated populations such as that of Faroe Islands or in virgin races where measles has not appeared for many years, or has been entirely unknown, that adults who have never had this disease are quite as susceptible as children.

Although in the examples which we have cited immunization follows a typical course of the disease, there is much evidence to show that inapparent infections frequently take place which nevertheless increase resistance. Such inapparent infections without much doubt are responsible for the well-known decrease with advancing age in the curves of Schick and Dick positive individuals,† although, as we shall see, Hirszfeld and others have offered a different explanation for these well-established observations. Increases in the natural or normal antibody titer with age have likewise been recorded both in animals and in man for pneumococcus (67), *H. influenzae* (68), *V. cholerae* (69), *Bact. dysenteriae* (Shiga) (70), poliomyelitis (71, 72), and the virus of swine influenza (73, 74, 75), and numerous other pathogenic and non-pathogenic organisms as well as for a variety of normal hemolysins which react with the erythrocytes of other species.

In attempting to evaluate the significance of those natural antibodies which react with pathogens whether these be filterable viruses or bacteria, in respect to the variation of the incidence of disease with age or indeed with the resistance of the individual in general, we must be cautious. Although the case incidence of diphtheria, scarlet fever, meningitis due to the influenza bacillus, and of poliomyelitis in general parallels the lack of antitoxic, bactericidal or virus neutralizing antibodies in the blood of

* For further information concerning placental transfer of immunity consult a paper by Mason, Dalling, and Gordan (65), and Needham's (66) monograph.

† For a discussion of the evidence for this statement see Chapters XIX and XXI.

the younger groups, there is no direct proof that a causal relationship exists between them, although this seems likely in some instances.

The relative lack in the younger age groups of antibodies other than those passively transferred has, as we have remarked, been usually explained on the assumption that overt or inapparent infection with the particular pathogen has not yet taken place. But an increasing body of experimental findings * suggests that other factors may be concerned. That the capacity to form antibodies is weak or absent in very young animals and only becomes fully developed as the individual grows older seems to be fairly well established. Moll (76) in 1908 found that rabbits three weeks old produced much less antibody when injected with a variety of antigens including serum globulin and *V. cholerae* than did adult animals. Freund (77), working with the typhoid bacillus as antigen, has more recently confirmed these results. Nattan-Larrier, Ramon, and Grasset (78, 79) have noted that young rabbits produce much less diphtheria antitoxin than older ones. In man, too, the production of antibodies appears to be less marked in infants than in older children, according to the observations of Frankenstein (80) and Halber and his co-workers (81) and a number of others. Such findings and many others which have not been cited would seem to indicate that the immunological mechanism in so far as it involves the formation of circulating antibodies responds less efficiently in young animals to equivalent antigenic stimuli. This phenomenon may well be related to the fact that as Lewis and Wells (81 a) showed, the blood serum of young animals is deficient in globulins which, as we shall see, are closely associated if not identical with antibodies, and at first they apparently lack the capacity to form these proteins.

The discovery that the antibodies concerned in the human blood groups are inherited and that these elements do not appear in a large percentage of infants until a variable number of months following birth, together with observations such as have just been mentioned on the differences in immunological behavior at various ages, suggested to Hirszfeld (82) that all natural antibodies might be governed by specific heritable factors and may develop independently of any contact with the antigen. He did not deny, however, that such contact might influence the formation of antibodies, although he considers the effect of external stimuli to be of minor importance. This author has come to regard antibodies as "biochemical organs" which may vary in their specificity and distribution among individuals according to the genetic composition of the latter. Like various anatomical structures the biochemical organs are supposed to mature at different periods during life. Since their maturation is assumed to be the result of the establishment of

* See Baumgartner for literature.

a biochemical reflex, no antibodies would result from contact with the antigen prior to the development of this reflex. In support of his theory Hirszfeld (83, 84) attempted to show that there is a relation between the inheritance of a particular blood group and immunity to diphtheria as determined by means of the Schick test. His results which do suggest a genetic linkage of this sort have, however, been questioned by a number of workers (85, 86, 87) and accordingly we must at present regard Hirszfeld's hypothesis as not proved, although by no means eliminated from further consideration, especially in respect to the origin of certain of the natural antibacterial antibodies, as contrasted with the antitoxins.

In addition to these views of Hirszfeld concerning the innate immunity of the individual little can be added which has not already been stated in our discussion of racial and species immunity. It may, however, be remarked that one animal will differ from another in respect to the ease with which it responds to active immunization as indicated by the quantity of antibody and the rapidity with which it appears. Thus it has long been known that individual horses vary greatly in their production of diphtheria antitoxin, and that the best producers are those which naturally possess a small amount of antitoxin in their blood. Recent studies by Stuart and his associates (88, 89) afford considerable evidence to show that the presence or absence of specific agglutinins for group A human red blood cells (see Chapter X) in the serum of normal rabbits is typically Mendelian in its inheritance. Furthermore they find, just as with the horses employed for antitoxin production, that the presence or absence of natural group-specific A agglutinins is a satisfactory indication of the ability or inability of rabbits to produce specific A agglutinins upon immunization. More data of this sort from breeding experiments would be desirable, particularly those based on techniques in which an antigen such as egg white was employed, since probably there would be present no natural specific antibody against this substance before immunization was begun. But these few observations strongly suggest that the capacity of individuals to respond to the parenteral presence of an antigen by the formation of an antibody is a manifestation of a physiological process genetically conditioned.

Additional host factors which are apparently related to individual variations in resistance have recently been described by Goodner and Miller (90, 91) and by Locke (92). In none of these studies is there any direct indication that the phenomena described are inherited characteristics, and on the whole this seems unlikely, although this possibility cannot be entirely eliminated. Goodner in his first experiments injected rabbits with Pneumococcus Type I and its homologous antiserum in such proportions that certain animals survived the infection, whereas others succumbed. He found that the heavier animals and those having high

white blood cell counts at the time of infective inoculation were able better to utilize the passively conferred immune principles of the antiserum. Continuing these investigations in mice, Goodner and Miller showed that the capacity of the animal to make use of the specific antibody could be correlated with the presence of relatively large numbers of white cells (monocytes) in the peritoneal cavity at the time the pneumococci were introduced into this site. In contradistinction to larger rabbits the smaller mice appeared to be more apt to survive the test dose of organisms and antiserum.

Locke has noted significant variations in the time required for the body temperature of individual rabbits to return to normal following immersion in cold water. The speed with which this "warming up" takes place runs parallel with the ability of the rabbit to resist intravenous infection with small numbers of virulent pneumococci. Since by various artificial procedures the "warming up" time could be lengthened or shortened with corresponding decreases or increases in resistance to infection, it is more probable that this type of resistance is associated with temporary alterations in metabolism than with innate differences.

There has been a great deal of confusion in regard to the significance of sex factors in susceptibility. That there are differences in the incidence of certain diseases of human beings in the two sexes at different ages cannot be questioned, but it is always difficult to disentangle the genetic factors from differences in exposure to diverse environmental conditions. Günther (93), after an analysis of the morbidity and mortality statistics of infectious diseases in different nations, states that he finds no evidence in support of Schiff's hypothesis which would regard susceptibility or resistance to certain infections as carried by a sex-linked chromosome. Since sex differences in the incidence of such infections as epidemic meningitis and pneumonia, which are more common in males than in females, or typhoid, scarlet fever, and erysipelas, which show the opposite distribution, are most definite during or after puberty, Hahn (27) conceives that the development of the internal secretions of the sexual glands may induce changes in the direction of resistance or susceptibility. A good example of a change of this sort is to be found in the marked resistance of female calves compared with the susceptibility of cows to infection with *Br. abortus* (94). In this case the alterations induced in the anatomical structure of the mammary glands and uterus at puberty appear to present favorable sites for the development of this organism. Aycock (95) has suggested that possibly the seasonal variation in the incidence of poliomyelitis might depend upon seasonal fluctuations in the secretory activities of the ductless glands. However, the analysis of the limited experimental work carried out on the possible effect of sexual

hormones on the resistance of laboratory animals to infection does not on the whole lend support to these theories. Dingle, Meyer, and Gustus (96), for instance, found no significant differences in the capacity of normal immature rats and those receiving estrogenic or gonadotropic hormones to form agglutinating antibodies for *H. pertussis*. Dingle in a recent unpublished survey of the literature on this subject was unable to find any conclusive data indicating a decisive participation either direct or indirect which could be assigned to the action of the secretions of the sexual glands. He concludes, however, that certain clinical and experimental results suggest a relationship of this sort.

The possible influence of diet on resistance to infection has of course been suspected by clinicians for many years. It is only comparatively recently, however — due largely to the discovery of the vitamins — that controlled animal experiments have been carried out.* Long ago, Theobald Smith suggested that dietetic factors such as the lack of green feed might account for an epidemic among laboratory animals which he observed. Since that time, this idea has been subjected to experiment, and the relationship between increased susceptibility to infection among animals fed on diets deficient in vitamins and possibly other factors has become more than a theory. Abel (100) in 1924 called attention to the frequency of secondary infection in scurvy, and Findlay (101) has found increased susceptibility to streptococci, pneumococci, and staphylococci in guinea pigs suffering from subacute scurvy. Others, like Coulthard (102) and Schiff (103), have obtained results with tuberculosis in guinea pigs which imply that vitamin C may be involved in resistance to this disease. We have referred in the previous chapter to the possible effect of vitamin C on resistance to small amounts of diphtheria toxin. But in spite of these reports and many others, we are still left in doubt as to the importance of vitamin C in immune responses. In the case of vitamin B and D deficiencies, there is, on the whole, even less indication that they are accompanied by an increased susceptibility to infection.

The work of a number of investigators (104, 105, 106, 107), however, has established the fact that animals fed on a diet lacking or grossly deficient in vitamin A are definitely more likely to contract spontaneous infection, and are also less resistant to inoculation than are controls kept under the same conditions but receiving an adequate supply of this vitamin.

The causes for the alteration in the immune state in vitamin A deficiency have not been precisely defined. Wolbach and Howe (108) noted marked changes in the epithelium of the nares, trachea, bronchi, and genito-urinary tract of avitaminotic rats. The normal type of cells

* Numerous references to the literature on the relation of the vitamins to infection may be found in the reviews by Robertson (99) and Clausen (97, 98).

characteristic of these sites was largely replaced by stratified, keratinizing epithelium. Siegfried (109, 110) studied vitamin A deficiency in chickens and confirmed the work of Wolbach and Howe and others. He found that there were changes in the mucous membranes of the respiratory tract and of the upper alimentary canal which consisted in a preliminary atrophy, following which there forms in place of the normal epithelium a stratified, squamous, keratinizing membrane, accompanied by a blocking of glandular ducts and sometimes necrotic changes. Siegfried attributed the increased susceptibility to the greater permeability of the changed portal of entry. Although this probably is a factor in the case of infectious agents which enter by these routes, it is clear from the experiments of Hotta (111), Lassen (112, 113), and others that animals deficient in the vitamin, even when artificially inoculated with bacteria by the intravenous or intraperitoneal route, which eliminates the effect of a susceptible portal of entry, show more fulminating and generalized types of infection. Accordingly, we must believe that some internal mechanism of immunity is impaired. In what this consists, we do not know. Studies (112, 113, 114, 115) of the capacity of deficient animals to produce antibodies following the injection of antigen have for the greater part shown that there is no impairment of this function.

Although it is well established that the lack of vitamin A leads to increased susceptibility, it is not evident that the administration of amounts of this substance greater than the minimum requirement raises the resistance above the normal. By means of the technique of the closed epidemic, Topley and his co-workers (116) were unable to find any increase in resistance to *B. typhi-murium* in mice fed on diets rich in vitamin A compared with those receiving much smaller amounts. Indeed, in one experiment the mortality was significantly higher among the animals given an excess of the vitamin. This unfavorable effect, however, may well have been due to other factors in the diet, as the authors point out.

Little attention has been directed toward the influence of dietetic factors other than the vitamins in respect to their influence on infections. That a deficiency of inorganic salts may render rats and mice more susceptible to a parasitic disease (*trypanosoma evansi*) and a bacterial infection (*B. typhi-murium*) is suggested by the experiments of Kligler and Geiger (117) and Hotta (111). Similarly, Watson (118) in a few experiments found that mice were more resistant when fed on a diet containing a mixture of alkaline salts than those which received the same diet with the omission of this component. This author has also shown that some factor, probably in dried milk, the nature of which is as yet entirely unknown, increases the resistance of mice both against infection with

B. typhi-murium and the endotoxin isolated from these organisms by the method of Raistrick and Topley.* Watson analyzed the earlier work of Webster (119) and Pritchett (120), on the basis of which those authors concluded that the superior resistance of mice to *B. typhi-murium* shown by animals fed on the so-called "McCollum diet" compared with those given the Rockefeller Institute diet was due to the greater amount of vitamin A in the former. Watson believes that their results are probably not to be attributed so much to the difference in the quantity of the vitamin as to the unknown factor in the dried milk which was included in the McCollum diet. To our mind, Watson's interpretation appears to be supported by the statistical analyses which he presents. It should be added, in respect to the studies of both Watson and Webster and Pritchett, that there was no evidence obtained which suggested that the increased resistance observed was in any sense specific. Moreover, it could not be accounted for, in Watson's experiments at least, by any change which rendered more difficult the passage of bacteria through the natural portal of entry.

Various other factors in addition to those which have been reviewed in the foregoing paragraphs undoubtedly may induce temporary changes in susceptibility in immune responses. We can here do little more than accord them brief mention.

A primary infection with one sort of pathogen often inaugurates a general depression of resistance, so that secondary invaders gain a foothold. It is a matter of common knowledge that simple coryza is not infrequently followed by serious disease of the respiratory tract caused principally by pneumococci and streptococci. The virus of human influenza seems to be particularly distinguished by its capacity to reduce the individual's resistance to pathogenic bacteria which invade the lung, with the production of pneumonias characterized by a very high mortality. In a similar manner, as Shope (121, 122) has shown, the virus of swine influenza permits the hemophilic influenza bacillus of swine to gain a foothold in the lung, with the result that the ensuing disease is much more severe than that which the virus alone is capable of inducing. Without this activating effect of the virus, the bacillus alone is entirely non pathogenic.

Not only do infectious diseases exhibit this tendency to reduce the resistance, but also non-infectious chronic conditions such as diabetes and nephritis.

Clinicians have given us much valuable information concerning the beneficial effect of rest and the harmful effect of fatigue in combating established infection. The data available from experiments in animals are conflicting, but those presented by Boycott and Price-Jones (123)

* See Chapters II and III.

on the influence of severe fatigue on the activation of a latent infection with *B. enteritidis* in rats established by feeding the bacteria support the clinical observations.

Sudden fluctuations in temperature and humidity have long been invoked as predisposing factors to infection. With chilling, there may be temporary congestion of the mucous membranes, due to vasomotor influences, which alter the secretions on their surfaces, and interfere with the normal mobilization of leucocytes, permitting penetration of bacteria which ordinarily would have been held back. Indeed, Hill and Muecke (124) have found that when individuals are placed in a warm, moist atmosphere, the nasal mucosa becomes infected and there follows the secretion of thick mucus. Transferred to a colder environment, the blood vessels of the mucous membrane become pale, but nevertheless remain edematous for some time. A physiological mechanism which may possibly account for the manner in which surface stimuli of various kinds could influence susceptibility may be found in the investigations of E. F. Müller (125, 126), who believes that many different kinds of non-specific stimulations of the skin may set up powerful reflex effects through the sympathetic nervous system. With this hypothesis in mind, he has injected intracutaneously relatively non-irritating substances like lactalbumin and distilled water, and has found not only a temporary disturbance of the distribution of the blood in the peripheral and splanchnic areas respectively, but a secondary change in the distribution of the circulating leucocytes. In the mechanism postulated by Müller, we may have an explanation not only for the reduction of resistance in mucous membranes due to temporary circulatory disturbances, but also a possible experimental basis for the therapeutic effects of surface applications such as wet dressings, heat, X ray, and counter-irritants.

That variation in susceptibility with the changing seasons of the year may occur is strongly suggested by the experience of numerous workers who have noted fluctuations in the manner in which animals responded to infection with a variety of pathogenic agents at different seasons.

Among the characteristics of the epidemiology of human diseases which were first recorded was that of seasonal periodicity. Hippocrates classified certain maladies noted as spring and winter ailments and others as belonging to the summer and fall. An enormous mass of statistical data compiled during recent times shows that the ancient Greek physician was entirely correct in his assertion. Such infections as poliomyelitis, pneumonia, and the enteric diseases, for example, exhibit characteristic maxima and minima during certain months.

The causes underlying the influence of seasons on infections are as yet ill defined. There can be no doubt that they are complex, and it is

obvious that here one cannot generalize. In the case of a disease like typhus, the severe conditions of winter favor the crowding together under circumstances in which the louse, which forms the means whereby transmission is effected, can easily pass from one individual to another. The aggregating tendency of cold weather may also play a role in the high incidence of respiratory infections during the winter and early spring by facilitating the exchange of infected droplets of mucus. Malaria in the temperate zones will only be passed by its anopheline vector from host to host during the warmer months when this mosquito is active. In addition to such purely external factors, there are many indications that the immune response, taking this term in its broadest sense, may be depressed at certain seasons and exalted at others. Including the possible changes which may be brought about by sudden alterations in temperature and humidity, and the fact that variation in the kind of diet consumed as well as in its content in vitamin A may take place with the revolving months, certain normal physiological processes have been shown to vary seasonally, such as the hemoglobin content of the blood, the respiratory frequency and depth, the alveolar carbonic acid tension, and the alkali reserve. Madsen (127), in an interesting summary of this problem, has recently suggested that these changes, which chronologically show maxima and minima coincidentally with the peaks and troughs of the incident curves for many diseases, may be tangible expressions of alterations in the mechanism of resistance. As far as fluctuations in the magnitude of the response of the antibody-forming apparatus go at different seasons, almost nothing is known. Madsen and Schmidt (128) have studied the capacity of guinea pigs to produce diphtheria antitoxin throughout the year and observed a very pronounced curve with a maximum in June and July and a minimum in the midwinter months. They likewise attempted to correlate the results of Schick tests in human beings with the seasonal incidence curve of diphtheria, but without conclusive results.

Since the quantity of ultraviolet radiation from the sun varies with the time of year, it has been urged that this phenomenon may be correlated with the seasonal incidence. But here again we have no conclusive experimental evidence which indicates that exposure to ultraviolet light renders human beings or the lower animals more resistant to infection. Thus Hill, Greenwood, and Topley (129) irradiated mice and then exposed them to contact infection with a mouse Pasteurella. No difference was noted between the irradiated animals and the controls. Colebrook (130) and Doull and his associates (131) found no significant difference over control groups in the incidence of measles, whooping cough, or colds in children and students who had been irradiated at frequent intervals throughout the periods of observation. Hornus (132), after reviewing

some of the literature on the changes in normal physiological processes which follow irradiation with the shorter waves, including ultraviolet and X rays, concludes that, although the former induce marked alterations in the chemistry of the blood, particularly in the levels of phosphorus, calcium, potassium, and cholesterol, it is impossible at present to assign to them any precise role in epidemiological phenomena.

Madsen concludes that the real stimulus for all such variations is the changing influence of the sun on the organism, but that the means whereby these changes are effected still remain unexplained. Certainly the great theoretical and practical implications of seasonal fluctuations in resistance should lead to more intensive effort to determine their causes.

This brief survey of most of the recognized factors, both extrinsic and intrinsic, which may influence the natural resistance of species and their component races and individuals will indicate how complex are the various mechanisms which are put into operation either through the contact of the organism with its surroundings or by the unfolding of physiological processes which arise as expressions of predetermined genetic factors. But from a subject which is admittedly perplexing and unclear in many of its aspects, certain basic principles have emerged. The immunity of strains and individuals belonging to a species susceptible to a given pathogen is preponderantly due either to passive immunity congenitally transferred or to previous infection with the same agent or possibly with one possessing antigens of similar or identical chemical nature. The newer data derived from experimental epidemiology show however that innate differences do occur. The fundamental elements in the mechanism of naturally acquired immunity are the antibodies and alexin of the body fluids and the phagocytic cells which may act conjointly with them in the destruction of the bacterium, virus, or other foreign materials of complex nature. These same defensive factors are also important and in certain cases probably essential elements in the immunity of species. The mode of their origin is here less clear. It may depend upon acquired immunity, but more probably, we believe, upon factors constantly inherited by all members of a species.

In addition to the cellular and humoral elements in natural immunity which were recognized almost from the beginning of the bacteriological era, many other non-specific entities, both in the internal and external, can unquestionably induce fluctuations in resistance. Climate, age, diet, temperature changes, and other physical and chemical agencies — their importance in immunity is clear, both from experimental and epidemiological experience. But their *modus operandi* remains largely obscure. Accordingly, in this volume we shall be dealing mainly with antigens and antibodies, and how by means of them we can convert susceptibility to resistance.

Artificial Immunization

The observations to which we have alluded concerning the effectiveness of the acquired immunity resulting from an attack by certain diseases upon members of a naturally susceptible species formed the point of departure for the study of the methods of artificial immunization, diagnosis by means of serological reactions, and specific therapy — techniques which in the main constitute the practical contributions of immunology. In the following pages we will present a general summary of the various means which have been developed by which immunity may be specifically induced or exalted, and outline the principal properties of the antibodies which appear in the body fluids in response to such measures.

Immunity after an attack of disease has been so striking that ancient observers, long before the birth of rational medicine, referred to it, and often drew conclusions of great hygienic importance. Thucydides, in the second book of his account of the Peloponnesian Wars, in describing the plague at Athens, notes the apparent safety from reinfection of those who had recovered, suggesting the possibility of their being therefrom immune against disease in general. The literature of the Middle Ages and of earlier modern times contains numerous indications that acquired resistance was clinically recognized as a result of recovery from many diseases. The phenomenon was put to practical use by the ancient Chinese and Indians. Thus the practice of inoculating children with smallpox material from the active pustules of patients, or making them sleep in beds or wear the shirts of sufferers was a dangerous practice but logical, on the reasoning that the disease conveyed to a person in full health and good condition would probably take a mild course and confer immunity, while the naturally acquired disease, contracted often because of the weak and debilitated condition of the individual, would be more apt to end fatally.

The first great advance which bridged the gap between these ancient observations regarding naturally acquired immunity and rational experimental immunization was made by Edward Jenner. It had been noticed before Jenner that milkmaids and others who had contracted cowpox in the course of their occupations were usually spared when a smallpox epidemic occurred in their community. Sporadic attempts had been made to put this observation to practical use, but no one had taken up the matter seriously. Jenner, interested by the reports of this nature and by his own observations, was impressed by the similarity between the local manifestations of smallpox, cowpox, and a disease of horses spoken of as "grease." Though at first disinclined to identify smallpox with cowpox (at present it is recognized by most investigators that the second is an attenuated form of the former), Jenner investigated cases of alleged protection by cowpox, a claim which before this had been hardly more than a rumor, and finally, with the encouragement of John Hunter, proceeded to the vaccination of human beings with cowpox, testing the immunity thus induced by subsequent inoculation of the same individual with smallpox. His report to the Royal Society in 1796 which was refused and his subsequent publications (133) incorporate the results of these experiments by means of which

the practice of vaccination against smallpox was introduced and the virtual eradication of the disease from civilized communities was attained.

The principles underlying smallpox vaccination are simple. The virus attenuated for man by passage through cattle incites a mild and localized form of the disease, from which the subject recovers rapidly. The recovery implies the mobilization of certain protective forces and a specific physiological alteration of the body in such a way that an increased resistance against the disease remains. In consequence, if the individual is subsequently exposed to spontaneous infection, his acquired specific resistance suffices to prevent invasion by the virus. This is merely an artificial imitation of the conditions which obtain when an individual recovers from an attack of a disease and is rendered immune thereby. In this case, however, the attenuation of the virus has eliminated the dangers attendant upon an actual attack. The immunity thus conferred is probably never as perfect nor as lasting as that following a seizure of the disease in its unattenuated form.

Pasteur's (134) fundamental immunological observations took origin in a purely accidental occurrence. Cultures of chicken cholera which had been allowed to stand without transplantation for periods of several months and then inoculated into chickens, failed to kill, giving rise in many cases to localized lesions only. It occurred to Pasteur that inoculation with such an attenuated culture might protect against subsequent infection with fully virulent strains and experimental investigation of this idea proved to be correct. He developed a method of "vaccination" against chicken cholera which consisted in injecting first an attenuated culture of the organism (*premier vaccin*), and, after 12 or 14 days, another less perfectly attenuated (*deuxième vaccin*), since he observed that a single inoculation was often insufficient to confer protection. After two inoculations a degree of immunity could be attained which sufficed to protect against spontaneous infection as well as against experimental inoculation with virulent germs.

These experiments constitute the true beginnings of the science, since, for the first time, an investigator working with a pure culture of a pathogenic microorganism had succeeded in conferring artificial immunity.

After his success in active immunization against chicken cholera Pasteur applied the same principles to anthrax. Successful attenuation was attained by a method which depended upon the cultivation of anthrax cultures at temperatures above the optimum for its growth. Toussaint (135) had shown that the resistance of sheep could be increased if they were inoculated with blood from animals dead of anthrax after this had been heated to 55° C. for ten minutes. Toussaint's idea had been that by heating the blood in this way the bacteria themselves

were killed. Pasteur (136) showed that this was not the case, but that what actually occurred was a reduction of the virulence of the strain by the exposure to heat. The method of Toussaint did not furnish a reliable means of attenuation, and Pasteur succeeded in developing a more satisfactory procedure as follows (137):

> Virulent anthrax bacilli were cultivated at 42° to 43° C. on neutral chicken bouillon. Under these conditions a gradual and progressive reduction of virulence occurs. After about 12 days of such cultivation the culture no longer kills rabbits, but is still virulent for guinea pigs and mice. After twenty-four or more days the virulence for rabbits and guinea pigs is lost and mice only can be killed with it. The latter — the most fully attenuated strain — was called *premier vaccin* by Pasteur, and, in the immunization of cattle or sheep, is first injected. After 10 or 12 days the stronger *deuxième vaccin* is administered.

This is the method which Pasteur used in his now classical experiments at Pouilly-le-Fort, in which he convinced a hostile audience of the efficacy of his immunization. Sheep were protected in the manner indicated, and 14 days after the last injection a fully virulent culture was inoculated and the animals found capable of successfully resisting it.

In the train of this work many other methods of producing active immunity have been devised — all of them of considerable theoretical interest and many of them practically adapted to some special case. We may conveniently classify these methods as follows.

Immunization with Living but Attenuated Cultures. (1) Methods in which the attenuation is obtained by heating. This is the method of Toussaint as outlined above, in which anthrax blood was heated to 55° C. for 10 minutes, and is probably the least efficient or reliable method for the attenuation of the anthrax bacillus.

(2) Attenuation by prolonged cultivation of the bacteria at temperatures above the optimum for their growth. This is illustrated by Pasteur's anthrax immunization as described in the preceding paragraphs.

(3) Attenuation by passage through animals. Examples of this are Pasteur's experiments with the "rouget" organism, in which passage through rabbits diminished the virulence for hogs. The attenuation of rabic virus for man by passage through rabbits is another instance, and Jennerian vaccination is also an example of this. Based on the same principle are Behring's (138) method now no longer used of immunizing cattle against tuberculosis by inoculating them with tubercle bacilli of the human type. Recently this method has been used successfully in the production of a vaccine against yellow fever by passage of the virus through the brains of mice and embryonic chick tissue (139).

(4) Attenuation by prolonged growth of bacteria on artificial media in the presence of their own metabolic products. This is the method first employed by Pasteur in chicken cholera, as described above. Calmette's (140) B. C. G. vaccine prepared for tuberculosis is based on the attenuation of the bovine tubercle bacillus by continuous cultivation in the presence of bile.

(5) Attenuation by drying. The classical example for this method is the Pasteur method of prophylactic immunization against rabies. Rabbits are inoculated with *virus fixe*, and their spinal cords dried for varying periods in bottles containing KOH at a temperature of about 25° C. The virus grows progressively weaker with each day of drying.*

(6) Attenuation by the use of chemicals. Chamberland and Roux (141) attenuated anthrax by growing it in the presence of various antiseptics. They used carbolic acid 1 to 600, bichromate of potassium 1 to 1500 and sulphuric acid 1 to 200, and found that, after a short time of cultivation under such conditions, the bacilli lost their ability to form spores and became avirulent for sheep. Behring (142) and others have applied this method to the attenuation of diphtheria toxin; Behring added terchlorid of iodin, Roux potassium iodid — iodin solutions, and Ramon (143) about 0.3 per cent formalin. The principle, of course, is not exactly the same in the last cases, since here the attenuation is not of the bacteria themselves, but rather of the toxin.

Active Immunization with Fully Virulent Cultures in Sublethal Amounts. The original methods of Pasteur carried out with chicken cholera and anthrax were aimed particularly at diminution of virulence, since these organisms, as isolated from the diseased animal, are so extremely infectious that it would be very difficult — in the case of many animals, impossible — to inoculate with the unattenuated germs without producing fatal disease. However, in the case of some other infections it has been found feasible to inoculate normal animals with the fully virulent germs in such small quantities that the body can successfully overcome them. Ferran (144) applied this method both to animals and to human beings with broth cultures of cholera spirilla. Högyes (145) introduced a similar procedure for immunization against rabies by injecting dilutions of fresh fixed rabic virus, beginning with a dilution of 1 to 10,000 and rapidly working up to a dilution of 1 to 10. In tuberculosis immunization with fully virulent cultures in small amounts has been attempted by Webb, Williams, and Barber (146), using the Barber method of isolation, and giving a single microorganism at the first injection.

In the general laboratory immunization of animals treatment with virulent bacteria in sublethal doses is of considerable value and frequently employed, but in human prophylaxis these methods are too dangerous to have practical value.

Active Immunization with Dead Bacteria, and Bacterial Extracts. Immunization with dead bacteria is the method which is most extensively practiced both in the laboratory immunization of animals and in the so-called vaccine treatment, both therapeutic and prophylactic, of man. It is of course most widely employed in prophylactic vaccination, such as the protection against typhoid fever, cholera, dysentery, plague, and many other diseases. Pfeiffer was one of the first to introduce this procedure and the methods of Kolle, Pfeiffer, and Marx in cholera were extended by Wright, Russell, and others to typhoid and paratyphoid infections.

* See Chapter XXVIII.

The literature of the past is full of technical modifications, most of which can at the present time be dismissed because we know, in the light of later investigation, that many of the methods by which the vaccines were produced rendered them useless.

In the production of antibacterial immunity, two fundamental principles revealed by the more recent studies on bacterial dissociation and the chemical nature of antigens must be observed. Neither of these had been regarded, of course, in many of the older methods, except those in which freshly prepared whole bacterial products were used, or in which recently isolated and virulent bacteria were killed by heat, formalin, or some other suitable bactericidal agent.

In our discussion of virulence, we have seen that when bacteria dissociate into the avirulent, rough forms they lose, temporarily at least, the capacity to synthesize certain of the antigenic constituents which are characteristic of the smooth, virulent forms. The type-specific carbohydrates of the pneumococci, for example, are not found in the rough variants and the toxic gluco-lipoids of the typhoid bacillus and certain of the salmonella are only present in the smooth organisms.* Because of the loss of these antigens, immunization with the rough forms induces either no immunity or only a feeble increase in resistance.

The recognition of the ease with which certain of these antigens † so essential for the inauguration of efficient immunization are impaired by relatively slight alterations in chemical and physical conditions, such as an increase or decrease of hydrogen ions, strongly emphasizes the danger of subjecting materials to be used as antigens in immunization procedures to manipulations which may reduce or destroy their effectiveness. Not only may we recall as examples the older findings concerning the striking effects of moderate amounts of hydroxyl ions followed by heating on the specific polysaccharides of Pneumococcus Type I and Type III, but we mention the recent work of Mudd and his associates (147, 148) on an important antigenic constituent of the hemolytic streptococcus which undergoes spontaneous inactivation mainly through oxidation within a few days following storage in the ice box. It is readily inactivated by heating at 56° C. for 30 minutes or exposure to a *p*H 10 for 10 minutes in so far as its capacity to combine *in vitro* with antibody is concerned. So far, no investigation concerning the behavior as an immunizing agent of the labile antigen inactivated in these ways has been reported, although it is clear that the material as prepared is a complete antigen. In this connection, the experiments of Dubos (149) on the effect of an autogenous enzyme on the antigenicity of pneumococci are also pertinent. This author found that this enzyme has

* See Chapters I and II.

† See Chapter II.

the capacity rapidly to alter the Gram-staining properties of the coccus so that under its influence in a few minutes Gram-positive cocci become Gram-negative. He then showed that these Gram-negative organisms had lost their ability to induce the formation of type-specific antibodies in rabbits. The action of the enzyme can be inhibited by moderate heating or low concentrations of formalin (150), and certain other chemical reagents. The relationship of these findings to the preparation of suitable suspensions of pneumococci and possibly other bacteria for immunization is obvious.

A number of examples of the application of these newer investigations on bacterial variation and the chemistry of antigens will be found in the chapters on the immunity to the various individual infectious diseases. Here it will be necessary only to reaffirm our original statement that there are two general principles which must guide the production of vaccines if successful results are to be obtained — the organisms from which the vaccine is made must be cultivated from a pure virulent or smooth type; in preparing the vaccine from such cultures, the organisms or their products must be so treated that no essential modifications in the chemical or physical character of the antigenic complex may occur. At present, most bacterial vaccines are prepared by simply killing a suspension of the organisms by means of moderate heating or by treatment with certain chemicals such as 0.2 per cent formalin, 0.5 per cent phenol, or 0.25 per cent tricresol. The present trend of our subject indicates that modifications in certain instances will surely be made in the methods now employed. With improvement in the techniques of the chemical isolation of bacterial antigens, it is perhaps not impossible that eventually we may have highly active, purified antigens which may be accurately standardized and preserved for long periods without loss of potency.

Numerous attempts have been made to vaccinate either with simultaneous injections of organisms and the homologous antiserum or with so-called "sensitized" bacteria — *i.e.*, organisms which have been exposed to the antiserum *in vitro* and thus have united with the antibodies contained therein. The general conception of the method was originated by Theobald Smith in connection with the production of diphtheria antitoxin in horses. It has been applied to active immunization with the bacteria themselves by Pfeiffer (151), by Metchnikoff and Besredka (152), and others. The latter immunized chimpanzees against typhoid fever with living, sensitized organisms. The method was further developed by Besredka, who recommended the use of sensitized, dead bacteria for the immunization of human beings, since he asserted that such vaccines were less toxic. Gay (153) extensively investigated the possibilities of the technique, but it has not supplanted the use of

killed, untreated organisms. Shiga (154) has applied the method of simultaneous injections in dysentery. The procedure has been frequently used in attempting to protect domestic animals against anthrax, symptomatic anthrax, and other diseases. Its most recent application has been to the preparation of a sero-vaccine against yellow fever by the workers of the International Health Division of the Rockefeller Foundation (155). Its use, however, has been abandoned in favor of a simple attenuated virus.

Active Immunization with Bacterial Toxins. As soon as the investigations of Roux and Yersin (156) had shown that in some diseases, at least, the injury sustained by the infected animal was largely due to the soluble toxins produced by the bacteria, it was logical to try to immunize animals with such products. The first attempts in this direction were those made by Salmon and Smith in hog cholera. The immunization practiced by Salmon and Smith was, however, a combination of immunization by bacterial products and by dead bacteria.

Similar attempts to immunize rabbits against certain forms of septicemia by the injection of culture filtrates were made by Chamberland and Roux in 1888 (157). However, neither in hog cholera nor in these other infections do the bacteria produce a true soluble toxin, and the immunization depended upon the injection of bacterial extracts. Nevertheless, these attempts pointed the way, and bore fruit in the investigations of Behring and Kitasato (158, 159). Fraenkel and Brieger (160), though following the method of injecting filtered diphtheria culture fluids, came to the erroneous conclusion that the toxin and the immunizing substances were not identical.

Behring, in collaboration with Kitasato, succeeded in immunizing animals with culture filtrates and with pleural exudates of diphtheritic animals. Similar results were accomplished with tetanus. Since the publication of these results toxin immunization has been effected in all cases in which a true soluble toxin can be demonstrated. The result of all this work has been the determination that susceptible animals may be actively immunized both against the effects of the toxin alone, as well as against the virulent bacteria themselves, by systematic treatment with culture filtrates containing the toxins.

Without doubt the greatest advance in the field of exotoxin investigation since their discovery was the recognition in the early years of the last decade of the value of formalin toxoid as immunizing agents.*

Artificial Passive Immunization. In the development of the fundamental facts regarding immunization, with attention focused early on the blood and body fluids as the probable carriers of immunity, it was a rational step from active immunization to the conception that such

* See Chapter III.

acquired immunity might be transferred from a treated to a normal animal by injecting blood from the former into the latter.

The first rational approach to this problem was made by Richet and Héricourt (161, 162), who immunized dogs against staphylococci, and attempted to transfer the immunity to normal rabbits by injecting defibrinated blood from the immune dogs. Their success was partial only for reasons that we will discuss directly. Reasoning similar to that of Richet and Héricourt was applied by Babes and Lepp (163) to rabies immunization. When the blood of rabies-immune dogs was injected into normal dogs and rabbits, and these inoculated with rabies several days later, the treated animals regularly survived the controls, but in one dog only was the occurrence of rabies absolutely prevented. The establishment of passive immunization as a scientific fact was finally accomplished in 1890–1892 by Behring and Kitasato (158). The results of this work — the direct outcome of their success in actively immunizing with soluble toxins, is summarized in their first paper as follows:

"The blood of tetanus-immune rabbits possesses tetanus-poison-destroying properties; these properties are demonstrable in the extravascular blood and in the serum obtained from this; these properties are of so lasting a nature that they remain active in the bodies of other animals, so that one is enabled to obtain positive therapeutic results by transfusing the blood or injecting the serum. These tetanus-poison-destroying properties are absent from the blood of non-immune animals, and when the tetanus poison is inoculated into normal animals it can be demonstrated as such in the blood and other fluids of these animals after death."

With these researches begins the practicable method of passive immunization which is now employed in the treatment of diphtheria, tetanus, and many other diseases of man and animals. The principle was also applied by Ehrlich (164, 165) to ricin and crotin immunity, in which he succeeded in working out passive immunization on a quantitative basis, showing that the degree of immunity in such cases could be directly referred to the amounts of the specific antitoxin present in the blood of the immunized animal. Calmette (166), and Physalix and Bertrand (167) then succeeded in producing passive immunization against snake venoms.

The strict specificity of these substances was from the first clear to von Behring and Ehrlich. It was also recognized from the beginning that passive immunity conferred by the antitoxic sera is almost immediately established and that proportionate increase of the amount of antitoxin can be produced against almost any amount of toxin. Behring also established the fact that the passive immunity conferred was of relatively short duration.

Passive immunization has achieved its greatest usefulness in the case of those diseases in which pathogenesis depends upon a true exotoxin —

which, as we have mentioned before, leads to the formation of an antitoxin in the immunized animal. The passive immunization is accomplished by transfer of the antitoxins from the treated to the normal animal.

In the case of bacterial infections in which no true toxin is formed — and the immunity depends, as we shall see, upon an enhancement of the bactericidal and phagocytic properties of the blood and the cells, passive immunization has not been equally successful. The probable reasons for this cannot be properly discussed until we have examined more closely into the mechanism by which the immune animal is protected after specific treatment with bacteria or their products.

The Phenomena Following Active Immunization. As a result of their studies upon the cellular and humoral reactions noted in naturally immune animals, Metchnikoff and his followers and the humoral school of Flügge and Nuttall were able to establish a certain degree of correlation between increased activities in phagocytic and bactericidal power and the increased resistance accruing from artificial immunization. Nuttall, for instance, found that the bactericidal properties of the sera of calves immunized against anthrax were greater than those exhibited by the sera of normal animals, and similar enhancement of the activity of the white blood cells was observed by Metchnikoff and his pupils in a variety of animals following vaccination with different organisms. But the subject remained vague and experiments inconclusive until the investigations of Behring and the others who discovered the antitoxins and the possibility of passive immunization. These findings threw an entirely new light upon immunological processes, and at once stimulated the investigation of the blood serum of immunized animals of all kinds for constituents that would react in the test tube with bacteria and their products or which would protect normal animals by passive transfer. The result was the discovery of the antibodies, the study of which forms the basis of most of our immunological knowledge. These factors which are to be found in the blood serum and other body fluids of certain normal, and almost if not all immunized, animals at least at certain times following the contact with the antigen, will be discussed in detail in the following chapters, but before we take up the properties of the individual reactions and the problems connected with them, it will be well to briefly survey the group of antibodies together in a purely introductory manner.

Bactericidal (Bacteriolytic) Antibodies. It is natural that extensive generalization followed the discoveries on the nature of antitoxic immunity. However, while it was found that the blood of all actively immunized animals possessed a certain degree of protective power for normal individuals, it was soon shown that this was not due in all cases to antagonism to the bacterial poisons. In immunity to the *Vibrio metchnikovi*

— in pneumococcus and cholera immunity — Sanarelli (168), Issaeff (169, 170), Pfeiffer and Wassermann (171), and a number of others showed that here, unlike diphtheria and tetanus, the protective power of the immune serum did not rest on "antitoxic" properties, but rather on antagonism to the bacteria themselves. It soon became definitely established that antitoxic immunity resulted only in the cases of those bacteria in which a true soluble exotoxin was produced, and where the disease following infection was primarily due to the absorption of these poisons.

The clearest definition of the conditions prevailing during immunization of animals with non-toxin-forming bacteria was that formulated at this time by Pfeiffer. The importance of the bactericidal power of serum, investigated by Flügge, Nuttall, and others, had dealt largely with variations of this general property, but had failed to recognize a specific increase in these powers during active immunization. Pfeiffer with Wassermann (171, 172) had studied the pathogenicity of cholera spirilla for guinea pigs, and had come to the conclusion that the animals died of toxemia (and not of bacteriemia, as claimed by Gruber and Wiener (173)), and that this toxemia was due to the liberation of poisons from the dead bodies of cholera vibrios, killed by the serum of the infected animals. Gruber and Wiener had shown that the injection of cholera spirilla killed with chloroform brought about a toxemia identical with that following inoculation with living cultures. They further determined that the resistance of animals against cholera was due to the bactericidal effects of the serum, which killed the injected cholera spirilla, and not to any poison-neutralizing property.

Issaeff (170), one of Pfeiffer's pupils, continuing this work, expresses Pfeiffer's conceptions as follows:

"Guinea pigs vaccinated against cholera, in spite of high immunity to infection with living spirilla, do not develop any immunity to cholera [endo]* toxins. The blood of immunized guinea pigs possesses no antitoxic properties. The maximal dose of cholera 'toxin' which immunized guinea pigs can withstand is not higher than that which can be borne by normal animals, and but slightly higher than the maximal dose of living spirilla, which they can survive. The blood of cholera-vaccinated guinea pigs possesses strong specific protective powers. The same specific immunizing properties are demonstrable in the blood of cholera convalescents toward the end of the third week of the disease."

This cleared the path for a definite conception of cholera immunity, which was formulated, in their next communication, by Pfeiffer and Issaeff (174, 175, 176). They showed that the cholera spirilla injected into the peritoneum of a cholera-immune guinea pig were subjected to a

* Bracketed word our own.

rapid dissolution, a process which could be observed by taking small quantities of exudate out of the peritoneum, at varying intervals, with capillary pipettes. No such dissolution occurred in normal pigs or with normal serum. But the same rapid swelling, granulation, and, finally, dissolution occurred when the spirilla were injected into the peritoneal cavity of a normal guinea pig, together with the serum of an immunized animal. The process took place without the co-operation of the leucocytes or other cells, and was specific. For instance, no "lysis" occurred when the vibrios "Nordhafen," "Massauah," and other cholera-like organisms were injected into cholera-immune pigs, but took place regularly when true cholera strains were used. The immunity of cholera-treated animals, therefore, was found to be an *antibacterial* and not an antitoxic one. Cholera spirilla introduced into a normal animal were permitted to multiply and accumulate until a sufficient number were present to furnish, upon cell death, a fatal dose of poison. In immunized animals the small quantities of bacteria first introduced succumbed rapidly to the lytic properties of the serum and accumulation was prevented.

By these experiments, now commonly spoken of as the "*Pfeiffer Phenomenon*," it was definitely proved that active immunization with bacteria incites in the serum of the treated animal a potent increase of bactericidal properties — an increase which is entirely specific in that the *bactericidal power* toward bacteria other than those employed in the immunization does not exceed the normal. The immunity in these cases, then, is not *antitoxic*, but rather "*antibacterial*," and depends on the development, in the immune sera, of antibodies, quite distinct from the "*antitoxins*," which act directly upon the bacteria themselves. These immune serum constituents were spoken of by Pfeiffer as "*bacteriolysins*" or "specific bactericidal substances."

Agglutination. Not long after the discovery of the specific bacteriolysins another property of immune sera was described by Gruber and Durham (177). They had been studying bacteriolytic phenomena with colon and cholera organisms, and noticed that these bacteria were agglomerated and gathered in small clumps when emulsified in homologous immune serum. Similar clumping had indeed been described before. Metchnikoff, Issaeff, Washburn, and Charrin and Roger had noted it on various occasions, but had not recognized it as a specific property of immune serum.* Gruber and Durham determined that it was present to a degree roughly proportionate to the degree of immunization attained, and that its specificity was such that it could be utilized for bacterial differentiation. They believed that the substances in the immune serum responsible for this agglutination were independent of other serum constituents and applied to them the term "*agglutinins*."

* For references see Chapter IX.

Primarily the phenomenon of agglutination was regarded as a part of the struggle of the body against the living bacteria and Gruber himself believed that it depended upon a swelling or "klebrig werden" of the microorganisms which tended to cause their sticking together, and rendered them more readily amenable to the action of the bactericidal powers of the serum. Bordet (178), however, early conceived the process as a physical phenomenon in which the bacteria themselves were entirely passive, and, indeed, Widal and Sicard (179, 180) soon demonstrated that bacteria killed by heat were equally as agglutinable as the living germs. Moreover, living bacteria agglutinated by means of specific antiserum in the test-tube are not killed. The present conception of the mechanism of agglutination is dealt with in a separate chapter.

Precipitation. These observations naturally suggested that the reaction between specific agglutinating serum and bacteria was based on individual peculiarities of the bacterial proteins, and it occurred to Kraus (181) to investigate whether or not the immune sera would cause any sort of reaction when mixed with the dissolved body substances of homologous bacteria. Working at first with cholera and plague, he obtained soluble constituents of the bacteria, both by allowing broth cultures to stand for varying periods and by emulsifying agar cultures in alkaline broth. The extracts were then filtered through Pukal filters to remove the insoluble materials. When the sera of immunized animals were added to these clear filtrates — cholera serum to cholera filtrate, and plague serum to plague filtrate, slight turbidity developed, followed within a few hours by the formation of small flakes. In other words, it was found that the mixture of a clear filtrate of a bacterial culture with the serum of an animal immunized against these bacteria resulted in the formation of a precipitate. The reaction was found to be as strictly specific as that of agglutination.

Although, from the beginning, Paltauf (182) had suggested that both phenomena of agglutination and precipitation might be due to the same factor in the antiserum, the property of precipitating homologous culture filtrates was attributed by Kraus and others to specific antibodies in the immune sera, distinct and independent of those previously described, and spoke of them as "*precipitins.*" There is much evidence to be presented subsequently which shows that the so-called complement-fixing antibodies and those that induce anaphylactic hypersensitivity as well as the agglutinins are probably identical with the precipitins.

Phagocytosis — Opsonins, Bacteriotropins. Throughout the years during which the attention of most investigators was concentrated upon the details of serum reactions, phagocytosis was continually being studied, since it became more and more obvious that destruction of bacteria within the immune animal body was in a considerable degree

due to the action of the circulating polymorphonuclear neutrophile cells, wandering cells, and certain fixed tissue cells of the reticulo-endothelial system. Indeed, it seemed quite clear, as a result of the investigations of Metchnikoff and his pupils, that in certain infections, such as those caused by the anthrax bacilli and other Gram-positive bacilli, the phagocytes were the most important in the destruction of the invaders. It was apparent, moreover, that in this increased engulfment of bacteria by cells, the antibodies of the serum played a role at first interpreted by Metchnikoff as a stimulation of the cells. It was not until relatively late in the development of immunology that this relationship was finally understood. The investigations particularly of Denys, Almroth Wright and his collaborators, and the Neufeld school made it clear that the degree of phagocytosis depended upon specific constituents of the serum, present in normal serum but increased in immunization, possessing all the attributes of true antibodies which united with the bacteria and thereby rendered them more easily engulfed by the leucocytes. These antibodies Wright called "opsonins," and Neufeld, thinking particularly of the phagocytosis-increasing antibodies of immune sera, "tropins."*

We have thus briefly reviewed the specific various properties which develop in the serum of an animal when it is systematically treated (actively immunized) with bacteria or bacterial products. The reactions which we have described, neutralization of poisons, agglutination, precipitation, and opsonic action have been ascribed, by those who discovered them, to specific serum constituents which are collectively spoken of as *antibodies*. It is a question still under discussion whether the various reactions are, as at first believed, due to a single antibody in each particular case — that is, a separate agglutinin, precipitin, or opsonin for each antigen — or whether, as we believe ourselves, these several activities are due to one and the same antibody which affects its specific antigen in such a manner that under different experimental circumstances it shows the phenomena described. This we will discuss in an ensuing chapter.

Antibody Formation Not Limited to Bacterial Antigens. Although we have used examples derived from experiments with bacteria or their products in the preceding survey of the antibody reactions, the property of inducing antibody formation is by no means limited to these organisms. As we have seen in the chapters dealing with antigens and toxins there are many substances both soluble and organized as cells which produce antibodies when introduced into the animal body.

The first observations of antibody production with non-bacterial substances were made independently by Bordet (183) and by Belfanti and Carbone (184) at almost the same time. They noted that the serum of

* See Chapter XI for references.

an animal injected with the erythrocytes of another species acquired the power of laking or dissolving these cells. That the normal serum of one species often reacts in this manner with the red blood cells of another species had long been known from the untoward results obtained in some of the earliest experiments on transfusion of blood. The phenomenon had been studied in considerable detail by Landois as early as 1875. The quantity of these normal "hemolysins" when they are present may be greatly increased following "immunization" with foreign cells. They are, of course, frequently absent in many normal sera.

The discovery of specific immune hemolysins, apart from its great intrinsic interest, profoundly influenced investigations on immunity generally, since it furnished a method of studying lysis which was more simple and more easily controlled than the analogous phenomenon of bacteriolysis. And since basically bacteriolysis and hemolysis are much alike, a great deal of our knowledge regarding the former process has been achieved through experiments involving the use of hemolysins. Possibly, however, reasoning on ground of results obtained with the latter system in respect to all the details concerned in the mechanism and conditions governing the bacteriolytic phenomenon may have been carried too far. For example, discrepancies have been noted by a number of workers, the most recent of whom are Fothergill and his coworkers (185), between the hemolytic capacities of complements derived from different animal species and their behavior with bactericidal antisera. These authors have presented a fairly complete review of the literature on the problem of the reactions of antibodies and complements derived from different species.

The specific hemolysins, then, are antibodies formed in response to immunization with red blood cells, analogous to the bacteriolysins. Because both are injurious to cells, we speak of them as cytolysins or cytotoxic substances. Their discovery naturally suggested the use of other kinds of cells, and the following years brought forth many reports of additional specific cytotoxins. In 1899 Landsteiner (186) and soon after Metchnikoff (187) described specific spermotoxins which appeared in the serum of animals treated with spermatozoa. Henle (188), who has lately studied the problem of the specificity of certain mammalian spermatozoa, after a review of the more recent literature on the subject and his own experiments concludes that not only are antibodies produced which react specifically with spermatozoa and not with the serum proteins but that it is possible to produce these antibodies in females of the same species, *i.e.*, he demonstrates the possibility of iso-immunization (production of antibodies in the same species from which the antigen is derived). Such claims must be scrutinized with care before being accepted, particularly when they assert the possibility of auto-

antibody formation. Von Dungern (189) in 1899 obtained similar reacting bodies by injecting ciliated epithelium from the trachea. Neisser and Wechsberg (190) produced leucotoxic antibodies by injecting cells of the granulocytic series. Many others have obtained similar results with these antigens. References may be found in the report of Chew and his associates (191) published in 1936. By injecting their antileucocytic serum into rabbits, these authors obtained a severe neutropenia which persisted for two days. Chew and Lawrence (192) have also obtained an antiserum which reacts specifically with lymphocytes, since upon injection into normal guinea pigs it produces a considerable lymphopenia which persists for at least 40 hours. Numerous authors have reported the production of specific cytotoxins against nearly all organs of the body.* It is clear that the attempts to invoke the role of cytotoxic antibodies in the production of various pathological conditions have been unsupported by satisfactory experimental data, since they were based on the observation of injury to organs following the injection of sera prepared by washed liver, kidney, pancreas, adrenal tissues, etc. In many of these cases it has been shown that the effect in part at least was dependent upon the hemolysins and agglutinins which induced the formation of emboli. Even today we doubt whether there is enough experimental evidence to warrant the conclusion that autocytotoxins play any significant role in pathological processes, but the idea is logical enough and should call for further experimentation.

The work outlined in the preceding paragraphs had thus extended the principles of antitoxin and lysin production beyond the scope of pure bacteriology, and had shown them to possess the significance of general biological laws. Similar generalization was soon possible in the case of the agglutinins and in that of the precipitins. In the former, the nature of the reaction limited it to observations upon cells in suspension and, in experiments upon hemolysis, it was discovered that the erythrocytes were often clumped when brought together with a hemolytic serum of moderate or feeble potency, or when solution, for other reasons, was delayed.

The first observations on the general significance of the precipitin reaction we owe to Tschistovitch (195) and to Bordet (196). Tschistovitch was studying eel serum which, as Kossel (197) had shown, is toxic for rabbits and causes hemolysis of rabbit erythrocytes. Its similarity to ricin stimulated attempts to produce an antitoxic substance against eel serum, even as Ehrlich had produced an antiricin. Tschistovitch observed that, when eel serum was mixed with the serum of a rabbit which had received several injections of this substance, the mix-

* For additional references see Chapter II; also Landsteiner's Monograph (193) (p. 63), and the 4th edition of this book (194).

ture became opalescent and soon a flocculent precipitate was formed. Coincident with this discovery Bordet made a similar observation. He had injected chicken blood into rabbits in the course of experiments upon hemagglutination. He found that the serum of the rabbits so treated acquired the property not only of producing hemolysis and hemagglutination of chicken cells, but also of giving a precipitate if mixed with chicken serum. Soon after this, precipitins were produced by injecting rabbits with milk (Bordet), egg albumen (Ehrlich, Uhlenhuth), and many other substances, and the specificity of such reactions was demonstrated by Wassermann and Schütze (198), Uhlenhuth (199, 200), and many others.

Tissue Immunity and Local Immunization

The science of immunity has developed pre-eminently through the study of the circulating antibodies which interacting with the invading parasites and their products either directly neutralize or destroy them or prepare them for ingestion and intracellular destruction by the phagocytic cells. Manifestly, since these soluble agents are carried throughout the body, the resulting immunity is distributed to practically all anatomical loci — this immunity is, then, a generalized state of resistance. But the thought has been in the minds of immunologists from almost the beginning that the cells of the body as a whole may undergo some change as a result of immunization whereby they are rendered less vulnerable to attack. We, therefore, have the conception of generalized tissue immunity independent of antibody mechanism. Others like Besredka have conceived of the possibility of a local tissue immunity depending upon the increase in resistance of certain types or groups of cells naturally peculiarly susceptible to various species of organisms.

A priori there are many reasons for admitting the possibility of some sort of immunity in which the recognized antibodies take no part. In the diseases in which the resistance appears to depend largely upon a persistence of infection such as tuberculosis or syphilis, the undeniable indications of alteration in the direction of greater tolerance, if not immunity, are so far as we know, not dependent upon the development of humoral factors. In some diseases such as typhoid fever or undulant fever an examination of the blood may reveal large quantities of specific antibody and yet the clinical course of the disease may show no immediate amelioration. Furthermore, recovery from a number of infectious diseases, as we have seen, affords a solid protection which may endure for many years — long after the circulating antibodies which appeared during or after the illness have disappeared. We have observed in Chapter I that there are differences in the ease with which various bacteria and viruses may gain access to the body through different

portals of entry. In all probability this depends in many instances upon such unfavorable environmental conditions as increased hydrogen ion concentration, the presence of proteolytic enzymes, and the mechanical action of washing away by saliva and mucus. But it is difficult thus to explain the striking resistance of the lingual and buccal tissues to infection even when they are badly traumatized or the relative ease with which invaders may multiply in the subarachnoid spaces or the peritoneal cavity contrasted with the more external tissues of the body.

In the case of bacterial allergy such as appears following infection with the tubercle bacillus and certain other organisms which will be described in detail in another place, we have direct evidence that many types of tissue cells become affected in some manner which is still unknown, so that they are seriously injured by subsequent contact with the protein derived from the bacteria. This protein has little or no effect upon the cells of the normal animal. Although it is not unlikely that this hypersensitivity may be mediated by a factor closely associated with the cell which is analogous to antibody, a constituent of this sort has not yet been demonstrated. It is, however, clear that an alteration in the reaction capacity of tissue cells may follow the introduction into the body of antigens in the form of bacteria. There is, moreover, considerable evidence which, in spite of its magnitude in the form of published papers, is not entirely conclusive, but which nevertheless strongly suggests that the antibodies are products of certain groups of cells, particularly those of the reticulo-endothelial system. The changes in cellular metabolism which lead to this production of antibody must be regarded as an indication that the cells concerned are not in the same immunological state as they were previous to antigenic stimulation.

Much of the experimental work on the problem of tissue immunity has been concerned with attempts to demonstrate and analyze the mechanism of local resistance. That certain areas or loci of the body can be made more refractory to infection than others can be accepted from the clinical observations on the course of erysipelas in human beings and the experimental work of a number of investigators such as those of Cobett and Melsome (201) and Gay and Rhodes (202), Rivers (203), and Amoss and Bliss (204). All these investigators employed rabbits as the test animal and obtained results which showed that following intracutaneous injection with living streptococci a local or a general increase in the resistance of the skin ensued, as demonstrated by subsequent reinoculation.

Although Gay and Rhodes, who found only a generalized increase in the immunity of the skin as a whole, were unable to demonstrate any acquired resistance following intravenous injection of the same organisms, Rivers and Amoss and Bliss showed that antibodies appeared in the blood with the inauguration of generalized dermal immunity. The

latter, however, did find that local skin areas became resistant before it was possible to detect antibody in the serum. Cannon and his co-workers (205, 206), who observed a local increase in resistance following dermal and intranasal vaccination, have carried out quantitative studies on the agglutinins developing locally in the skin and mucous membrane after the introduction of killed *Bact. paratyphoid B.* Since they found agglutinins in somewhat greater concentration in the vaccinated loci than in the spleen and liver, they conclude that antibodies are formed at these points. Although we believe this interpretation is open to question, their findings do indicate that antibody tends to be present in slightly larger amounts in areas which have been brought directly in contact with the bacteria.

Beginning with the report of Pfeiffer and Issaeff (207) in 1894, who observed a marked increase in the resistance of guinea pigs to cholera spirilla injected into the peritoneal cavity which had previously received an injection of sterile broth, a number of workers have noted that preliminary local treatment with a variety of non-specific reagents capable of inducing an inflammatory response will tend to exalt the resistance of an animal. Gay and his co-workers (208, 209) and Menkin (210, 211) have in recent years studied this phenomenon intensively. According to Gay the macrophages which enter late in the process comprise the essential factor in this type of immunity which he terms tissue immunity. He does not deny that the efficiency of these cells may be increased by the presence of specific antiserum, but he considers them to be quite capable without its aid of disposing of certain organisms such as the streptococcus. Furthermore, he believes that through immunization a mechanism may be introduced by which these cells respond by a much more rapid mobilization and perhaps by a specifically increased metabolism when the same bacteria again enter the body.

Menkin has shown that in inflammatory sites bacteria, inert particulate substances, and soluble materials tend to become fixed or localized, at first mainly by the plugging with fibrin of the small lymphatics draining the area. This mechanism, which is entirely non-specific, is particularly effective in hindering the dissemination of certain bacteria like the staphylococcus. Others, such as the streptococcus, probably because they do not cause an injury so intense or immediate, are not so effectively blockaded within the inflammatory focus. No doubt there are other factors in the complex processes of the inflammatory response which may play a role in the defense against invasion which it unquestionably affords.

With these considerations in mind we may proceed to an examination of Besredka's (212, 213) views on local tissue immunity, since it is he that has departed most widely from the traditional conception of the

nature of immunological responses. Starting with the observations relative to the selectivity for certain portals of entry exhibited by various bacteria, Besredka reasoned that an organism like anthrax, which in many instances enters the body through the skin, encountered there susceptible cells with which it reacted to produce substances that inhibited the activity of the mobile phagocytes. According to him only the cells of the dermis are "receptive" in this sense in so far as the anthrax bacillus goes. Therefore, if inoculation is effected by a route which avoids contact with these susceptible cells, no disease will result, since the organisms will be rapidly destroyed by the mobile phagocytes. Immunization according to the theory would consist in rendering the receptive cells resistant by "desensitizing" them with the essential products derived from the bacteria in a manner analogous to desensitization of the anaphylactic state. These cells would then fail to react upon subsequent contact with the organisms and thus no antiphagocytic substance would be formed. Once this local immunization was accomplished the whole animal would be immune because all but these cells are assumed to be naturally resistant or non-receptive. Moreover, since the guardians of the portal of entry have been rendered insusceptible, in most cases invaders will be repelled at the outset and never gain access to the interior of the body. In support of this theory he presented the results of experiments on dermal anthrax vaccination of guinea pigs, as well as many others based on the feeding of large amounts of organisms such as typhoid, dysentery, and others of the enteric group, which he asserted, induce a local immunization of the receptive cells of the intestine when administered in this manner. He applied the same method to the oral vaccination of man. There can be no doubt, not only from his results but also from those of others, that a certain degree of immunity may be obtained by these procedures. Besredka's interpretation of the findings has however met with skepticism. Thus, in experiments carried out in our laboratory, the occasional immunizing effects through feeding of bacteria, though irregular, were found to be definite, but we felt it quite impossible to determine whether the occasional immunity obtained was due to a local immunization in the sense of Besredka or to a general increase in resistance consequent upon absorption from the bowel. The latter, which we believe was the case because of the development of agglutinins, would indicate that vaccination by mouth was nothing more than a very unreliable and quantitatively uncontrollable variant of subcutaneous injection. As far as the application of this method to human immunization is concerned, we have as yet insufficient data to judge of its effectiveness. It is noteworthy, however, that Park and his co-workers (214, 215), who have of recent years been engaged in investigating the value of the so-called B.C.G.

vaccine against tuberculosis, have abandoned the method of oral administration recommended by Calmette in favor of the subcutaneous route of injection. Besredka has also applied his theory of local skin immunization to staphylococcus and streptococcus disease in which he has employed a treatment which consists in the topical application of dressings saturated with filtrates of cultures of these organisms with the object of desensitizing the receptive cells with the "antivirus," as he calls it, *i.e.*, the substances which in the intact bacteria are able to react with these cells and which are present as excretions or extractives in the filtrates. Experimentally, infiltration of the skin does exalt the resistance of the area to a certain degree, but from the results of a number of workers, including Mallory and Marble (216), Toomey and Friedlander (217), and others, it would seem that the effect is entirely non-specific, since they could reproduce the phenomena with sterile broth, which is a mildly irritating non-specific incitant of inflammation. The basis for this immunity is without much doubt largely dependent upon the non-specific defensive properties of the inflammatory process itself which we have mentioned above. Similarly, Besredka's contention that the skin alone is susceptible to anthrax infection has not been supported by the experimental findings of a number of workers. Sobernheim and Murata (218), for example, found that the dose of organisms required to kill a guinea pig was smaller when the subcutaneous route was employed than when intradermal inoculation was carried out.

Considering all the available evidence, we feel that it fails to demonstrate a new and heretofore unrecognized immunological mechanism which depends upon the development of a local refractory state in the tissue cells themselves, and is independent of antibodies, phagocytosis, and the anti-infective functions of inflammation. But it is apparent that different areas of the body may show variations in their capacity to resist infection. Such resistance may be either naturally present or induced as the result of various procedures which may lead to a temporary immunity. This may be, as we have stated, largely the result of non-specific factors, or, in certain cases, the result of the localized formation or appearance of specific antibody. And finally it is exceedingly doubtful that local resistance is associated with any one class of cell but is, rather, a property of the area as a whole.

In spite of these considerations, we would not hastily discard the general conception of an immunity of tissues independent alike of antibodies and phagocytes, since there are indirect indications that this may exist. We really know nothing of its nature, however, and before we can accept it as a basic immunological fact, we must await the result of further experimentation, which, it seems to us, will depend upon the discovery of new techniques.

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CHAPTER V

ANTIGEN-ANTIBODY REACTIONS. NATURE OF ANTIBODIES

General Considerations. In preceding chapters we have outlined the responses of the animal body to immunization with the various substances spoken of as antigens and have defined the chemical and physical nature of these substances. From a broad biological point of view, we may say that the one basic fact upon which all the rest of immunology is founded is the power of certain materials to elicit a specific change in the cellular reaction capacity.

It is reasonable to assume, as Ehrlich did, that *the tissue cells of higher animals are normally prepared only for the metabolic processes concerned in the nutritional and excretory functions essential to the maintenance of life*, the liberation of energy, and the performance of special secretory functions peculiar to their activities in the general body economy. Compared with the cells of the simpler forms of life, the normal functions of the mammalian tissue cells are considerably more specialized. A single-cell protozoan, for instance, may take up boiled starch granules or bacteria and deal with them, digesting and extruding waste products. In only a few cells of the body of the higher animals such as the phagocyting ones, does such an atavistic ability to deal with varieties of substances unprepared by digestion survive.

The essential consideration is that *in the bodies of the higher animals the substances which reach the cells for nutritional purposes through the blood or lymph, or perhaps through the intercellular fluids beyond the lymphatics, come into actual cellular contact only after elaborate preparation by preliminary digestion.* Thus, proteins reach the ultimate cells which they nourish only in the forms of amino acids; the fats are in the form of glycerol, fatty acids, and soaps, and the carbohydrates in that of simple sugars. The cell is thus normally prepared to deal only with substances in the chemical and physical conditions into which preliminary digestion has put them. It seems to be a biological principle that most materials which are not in this chemical and physical class of predigested nutritive matter give rise to a specifically altered state of reaction capacity on the part of the cells. Thus, even when dealing with substance as low in molecular structure as alcohol, some of the narcotics, quinine, morphine,

and other alkaloids, etc., the facts of habituation and idiosyncrasy indicate that such altered conditions have been produced. And such drug tolerance or habituation cannot always be explained entirely by increased powers of excretion or destruction. For, although increased eliminating properties are involved in the process, still it has been shown, at least in the cases of alcohol and morphine tolerance, that equal concentrations in the blood stream of normal and of tolerant individuals may cause a deeper state of intoxication in the abstainers than in the habituated. (Rubsamen, Van Dongen, cited from Wells.) Thus, it is necessary to assume, as Wells puts it, "a certain refractoriness or cellular immunity in addition." It should be stated in this place, however, that while it has been possible to harmonize *drug idiosyncrasies* with truly immunological forms of hypersensitiveness, we have not as yet any reliable clues by which an immunological mechanism for *drug tolerance* can be postulated.

When we consider the varied chemical and physical constitution (1) of the many substances which may come into contact with the body cells, from the drugs at the bottom of the list to unchanged proteins, it need not astonish us if the manifestations accompanying the development of such susceptibilities or tolerances are subject to a wide range of variations. Common to all of them, whether it is the fluctuating tolerance to morphine or the quasi-permanent acquired immunity to plague, cholera, or typhoid, is the fact that the tissue cell is basically changed in its reaction to the particular material concerned with a degree of specificity which seems to be more striking as chemical complexity of the foreign substance increases. Important differences, however, exist in the laws governing the acquisition of the altered cell reaction capacity and in the manifestations by which they may be recognized.

Ehrlich conceived the cell as a *giant molecule*, a homogeneous chemical system with an enormous molecular weight, provided with a large number of "side chains"; these "side chains" were conceived as normally adapted for union with the various types of foodstuff that might be brought to the cell in the course of nutrition, but also capable of reacting with other substances reaching the cell under unusual conditions. Since his time the conception of the cell entity has changed considerably. The cell can no longer be regarded as a large molecule of living matter, the life of which is maintained by a constant interchange of chemical unions and dissociations, but must be conceived as a heterogeneous series of phases separated from each other by semipermeable surface layers, resulting from the concentration at the surfaces, particularly of lipoidal substances. But the semipermeable membranes which separate the internal subdivisions of the cell and delimit it from its environment probably do not consist of lipoids alone, but are composed of complex

colloidal intermixtures of lipoids and proteins (2). It is clear that the reaction of an extraneous substance with a cell must depend not only upon its chemical but also upon its physical properties, according to which it may enter the cell and react with the substances in its interior or may come into contact only with the outer-surface phases of the protoplasm. It is probable that the permeability of the cell membrane may change under various conditions, and that in the problems of hypersusceptibility a part of the process may consist of an increased ability of the injurious substances to get into the cell.

Thus, in reconstructing our ideas of the changes which may take place in cells under conditions of immunization or sensitization, we must include the physical properties of the foreign substances concerned. The thought suggests itself strongly that nondiffusibility, the property of reacting only with cell surfaces, and antibody formation are in some way connected.

The process of antibody formation is not at all limited to infections, but is of general biological significance in connection with the reaction of the body to contact with "antigenic" substance.

We must look upon the entrance of bacteria into the body as the invasion of a foreign antigenic substance which differs from the others only in that it is self-multiplying and has developed certain localization affinities for individual organs and tissues. The latter, together with the pharmacological action of specific poisons, largely determine the symptomatology of the respective disease. As antigens, however, the bacteria do not differ in principle from other foreign protein substances.

The Location of Antibody Formation. The tissue cell as the ultimate functional unit must be looked upon as the source from which originate the various protective constituents of normal and immune sera.

Pfeiffer and Marx (3) exsanguinated animals five days after injections of dead cholera spirilla and found that at this time bacteriolytic antibodies were more concentrated in the spleen than in the blood serum itself. Wassermann's (4) analogous experiments with typhoid bacilli seemed to show a higher antibody content in spleen, bone marrow, thymus, and lymph nodes than was present in the blood at an early period of immunization. Although these investigations, as well as those of Castellani (5), seem to indicate association of the special lymphatic organs with antibody formation (6), extirpation of the spleen (7) before immunization has not prevented animals from responding to injections of bacteria and red blood cells with antibody production. The experiments of Deutsch (8), in which reduction of antibody formation resulted when splenectomy was practiced three or four days after immunization was begun, cannot be accepted as conclusive, since operation or interference with normal functions during the active immunization would naturally lead to a less perfect response.

Experiments which have some significance in indicating that antibodies can be formed in many different parts of the body are those of Wassermann

and Citron (9), who injected typhoid bacilli into rabbits by the intraperitoneal, intravenous, and intrapleural routes and, nine days later, produced aleuronat exudates of pleura and peritoneum in these animals. They then compared the bactericidal strengths of blood serum and of the exudates, finding that the concentration of antibodies was highest in the blood serum in the intravenously inoculated animals, but highest in peritoneal and pleural exudates, respectively, in the animals immunized by injection into these cavities. Again, they injected typhoid bacilli subcutaneously into the ear of a rabbit, ligated the ear immediately at its base, and kept it so for several hours. After nine days the bactericidal titer of the blood serum was determined and the ear amputated. They report finding an immediate drop of antibody contents after amputation. Experiments of similar significance have been carried out by Römer (10), who instilled abrin into a rabbit's eye and found that the retina of the eye developed an antitoxic power against abrin which protected mice, while the retina of the other eye remained negative. Experiments such as these indicate that antibody formation can take place throughout the body and is to some extent determined by the location in which the antigen is administered. It suggests the possibility of local immunity in special tissues. Besredka believes that in intestinal diseases such as dysentery, typhoid fever, cholera, the significant immunization is in the mucosa of the intestinal canal, while in diseases like anthrax it is the immunization of the skin which is significant. Similar suggestions had been made long before by Wassermann and had been applied to smallpox by Kraus.

Clinical observation in a number of diseases suggests a similar conception. The most obvious example is that of erysipelas, where the infection may progress along the edges, while the central area of infection is returning to normal. In adults it rarely happens that the process extends back into the originally infected area, although this may happen in children.

In infections in which a considerable part of the immunological mechanism depends upon the activities of fixed tissue cells, as probably in tuberculosis and in syphilis, local immunity of tissues which have reacted is probable, though it is difficult to prove. Suggestive in this regard is the fact that circulating antibodies can be more actively produced in tuberculous than in normal animals.

There has been a great deal of speculation as to the role of the reticulo-endothelial system, in connection with antibody formation. The cells of this system, as Aschoff (11) has pointed out, fulfill the criteria necessary for the assumption of antibody-forming functions of wide distribution throughout the body — in capillaries, in lymph spaces, and in connective tissue. Investigations to demonstrate the connection of these tissues with antibody formation have consisted of attempts to block the capillary endothelium with various colloidal substances, then measuring the relative powers of animals so treated to form antibodies as compared with normal animals. Such work was begun by Standenath (12) and by Vanucci (13). The former used India ink to block the reticulo-endothelium in rabbits and then tested the capacity of these animals for precipitin formation. His results indicated an increase of antibody formation rather than a decrease. Vanucci, using carmine and Wasserblau, found a decrease of agglutinin formation. Gay and Clark (14) saturated rabbits and rats with trypan blue by daily intraperitoneal injections, over a period of two weeks, fol-

lowed by decreasing amounts of dye administered daily throughout immunization with washed sheep's blood. They believe that blocking, if sufficiently and properly done, may almost completely suppress antibody formation. Others have not been able to repeat these results. It cannot be concluded with any certainty today that blocking has proved the reticulo-endothelial system to be the site of antibody formation. Indeed, it is not impossible, assuming final confirmation of results like those of Gay, that the blocking may merely interfere with the proper absorption of the injected antigenic substances. Jungebluth and Berlot (15), studying the complement titer after blockade of the reticulo-endothelial system with India ink, showed that there was a drop in complement contents, which attained its maximum in three hours and lasted for about twenty-four, but showed at the same time that the blocking caused a marked impairment of the respiration of the cells of liver and spleen during the first eight hours after the India ink injections. In other words, there was functional impairment of the organs, secondary to endothelial blocking.

Carrel and Ingebrigtsen (16) attempted the production of antibodies in tissue cultures. They cultivated guinea pig bone marrow and lymph gland tissue in guinea pig plasma and washed goat corpuscles and found that substances were generated in the cultures after five days which were hemolytic for the goat corpuscles. However, the fluid hemolyzed the cells without the addition of complement.

Antibodies and Internal Secretions. A great deal of work has been done on the possible relationship between immunity and the organs of internal secretion. A great many of these investigations have concerned themselves with the relationship of internal secretions to the formation of alexin, and have no direct bearing on antibody formation. Louise Fassin (17) reported that subcutaneous injection and feeding of thyroid extract in dogs and rabbits was followed by a rapid rise of alexin contents of the plasma. Removal of the thyroid gland in rabbits was followed by reduction of alexin. These experiments are not conclusive, since the effect may have been indirect, due to the influence of the thyroid gland upon metabolism in general. Marbé (18) associated the thyroid gland with opsonin production by work analogous to that of Fassin. The liver has come in for considerable attention in the same connection. Morgenroth and Ehrlich (19) noticed diminished production of alexin in phosphorus-poisoned dogs. Nolf (20) extirpated the liver in dogs but, obtaining irregular results in these animals, carried out similar experiments in rabbits with Eck fistula. Since the life of the animals after operation was only three or four hours, Nolf's observation of the rapid drop in alexin has little value.

Studies on the effect of adrenal secretions upon antibody formation have been more significant. That adrenalectomy lowers resistance of animals against toxins and drugs, as well as bacteria, has been frequently observed (21, 22). In 1913 Munk (23) showed that the immunization of animals to toxins was often accompanied by hyperplasia of the adrenal

cortex, and increase of the characteristic lipoids; and Mulon and Porak (24) noticed similar hyperplasia and increase in cholesterol of the adrenal cortex during the immunization of animals with a variety of antigens. The literature on the subject indicates rather consistently that there is some relationship, either direct or indirect, between the suprarenal glands and the physiology of antibody formation. Quite recently, Fox and Whitehead (25) showed that the administration of cortico-adrenal extract to normal rats immunized with a single injection of sheep cells resulted in an increase of 60 to 70 per cent in the hemolysin titer of the injected over the control rats. Prolonged administration of the extract, together with repeated sheep cell injections in rabbits, produced increased titers, especially during the early stages of the process. The mechanism of the effect is still obscure.

Normal Antibodies. The serum of normal animals and human beings contains antibodies which are in many respects analogous to those induced by the injection of blood cells, bacteria, and other antigens. There are, however, certain differences between these normal substances and those immunologically induced which indicate that the two may not be identical. Specific absorption of normal antibodies, such as, for instance, the agglutinins for red blood cells of another species which may be present in the serum of a given animal, removes the particular antibodies, but the union is not nearly as firm nor as specific as that which takes place when immune antibodies are similarly absorbed. Cells that have been agglutinated by normal serum, when moderately heated in salt solution, give up a large percentage of the agglutinins, and such liberated normal agglutinins may act on a variety of other types of red cells.

Landsteiner states the case by saying that normal serum agglutinins are specific only in so far as they react to a different degree on various cells.

As far as the origin of normal antibodies is concerned, there are two obvious possibilities. One is that these antibodies are not really normal but are developed in the course of life by spontaneous immunization — that is, contact either with the specific antigen against which they are formed, or with antigenic substances that chemically overlap with the ones on which the serum is found to react. This is undoubtedly the case with many so-called "normal" antibodies, especially in connection with phenomena like the sheep hemolysins which may appear in rabbits as a consequence of invasion with bacteria like *Bacillus lepisepticus* which contain Forssmann antigen. It is supposed, however, by Landsteiner that these immunologically acquired antibodies do not account for all so-called "normal" antibody. Landsteiner believes that such accidental immunization is less important than the physiological, genetically determined formation of antibodies. Hirszfeld has been one of the chief

exponents of this point of view. The best example is the regular and hereditary presence of iso-agglutinins in human serum, and it is well known to all who have studied the toxic action of the normal serum of various animals that hemolytic and hemagglutinating properties for other animals are often species characteristics. We must assume, therefore, although the problem is not by any means solved, that substances not identical with but closely analogous to immune antibodies are genetically developed in certain animals without any relationship to previous immunological contact. It has been suggested by such work as that of Jungebluth that such development, occurring at or about puberty, may account for the increased resistance of adults to some infections to which infants are highly susceptible. It is obviously very difficult, in view of the innumerable possibilities for contact in human communities, to come to any decision about this matter until a great deal more well-controlled experimental work has been accomplished.

Mechanism of Antibody Formation. When we consider the production of a specific antitoxin in response to the treatment of an animal with a toxin, it is but natural that Buchner and others should have at first assumed that the antitoxin is, in each case, a product obtained by the action of the body tissues from the toxin itself. Such a simple conversion of toxin into antitoxin, however, was questioned because the amount of antitoxin incited in the immunized animal is out of all proportion great in comparison with the amount of toxin injected. Thus Knorr (26) found that 100,000 units of antitoxin may be produced by the injection of the toxin equivalent of one unit. Salomonsen and Madsen (27) observed that pilocarpin injections will increase the amount of antitoxin produced by an animal, which pointed to the likelihood of the participation of the general physiological activities in the production of antibodies. The experiments of Roux and Vaillard (28), in which antitoxin production in immunized animals continued even after the entire volume of blood had been removed by repeated bleeding, rendered the Buchner idea quite unlikely. Nevertheless, the conception that antigenic elements entered into the structure of the antibody was, later, revived by Manwaring.

Conclusive refutation of this assumption that parts of an antigen may enter into the composition of an antibody was finally made possible by experiments in which compound proteins containing easily identifiable elements were used. In the work of Doerr and Friedli (29) protein-arsenic compounds were used as antigens, and the antibodies in the resultant immune serum contained no excess of arsenic over the sera of normal controls. Similar evidence was obtained by Hooker and Boyd (30). The latter, comparing the amount of antigen used with the combining amounts of antibody produced, found that a single antigen mole-

cule may give rise to several hundred antibody units. Their calculations confirm the opinion of Heidelberger and Kendall (31), based on similar evidence with another system, that the antibody yield is so greatly in excess of the injected antigen that it is quite out of the question that every antibody unit should contain portions of the original antigen.

Another current theory was that known as Ehrlich's *"Side Chain Theory."* Briefly outlined, this conception was as follows: The reactions of immunity represent a repetition of the processes of normal metabolism, and their adjustment to new conditions is only another phase "Uralter Protoplasma Weisheit" (32). It is impossible to conceive the nutrition of body cells without assuming that the assimilable nutritive substances come into physical and chemical relationship with the protoplasm of the cell. Considering the variety of substances which may thus be brought into contact with cells in normal and abnormal metabolism, the body cell must possess a variety of atom groups, by means of which it can unite with these substances to assimilate them. In order to enter into similar relationship with toxins and other antigens, the cell unites with the antigenic substance and either assimilates it without sustaining harm or is injured in the process.

The living cell is conceived as consisting of a central chemical nucleus, the "Leistungskern," more or less stable, in that the specialized tissue function is dependent upon it, and a manifold variety of "side chains," atom groups, by means of which it can enter into relationship with the materials carried to it. Thus we may conceive the "Leistungskern" as the central carbon ring, as, for instance, in salicylic acid in which the hydrogen atoms, the hydroxyl, and the acid radicals represent "side chains." By means of the latter, the compound can enter into relation with other substances, as, for instance, with CH_3 in the formation of methyl salicylate. The cell, therefore, is looked upon as an active chemical complex, retaining its own peculiar functional characteristics by reason of the "Leistungskern," but constantly getting rid of waste products and entering into new union with extraneous materials by virtue of its "side chains." These side chains are spoken of by Ehrlich as "receptors." Pasteur noted the fact that yeast ferments could decompose dextrorotatory tartaric acid while they did not affect the levorotatory variety, and Emil Fischer (33) showed that only those carbohydrates possessing 6 and 9 carbon atoms were subject to fermentation by yeasts, and of these only the ones belonging to the "d" series. Landsteiner's recent demonstration of the dependence of the antigenic specificity upon the asymmetric carbon atom in tartaric acid-protein linkages points in the same direction (34). In the case of the absorption of substances belonging to the class of antigens, foreign proteins or toxins, the receptors occupied by these substances are rendered useless to the

cell and, if the cell continues to live, must be regenerated. If the antigen is repeatedly thrust upon the cell, the process of regeneration may be not only sufficient to compensate for the loss of the eliminated receptors, but may follow the general law of overcompensation formulated by Weigert, and receptors of the variety occupied by the antigen are produced in excessive number.

Thus the restitution of cell receptors far exceeds the quantity normal to the cell and may proceed to such a degree that the cell, becoming "top-heavy" with these elements, sloughs them off into the surrounding lymph and blood, where they circulate as free receptors. These free receptors, then, having specific affinity and combining power for the antigen which incited their production, represent the circulating antibody.

In summary, it seems impossible, in view of the quantitative relationships discussed, to assume that antigens actually enter into the composition of antibodies. Moreover, such a hypothesis cannot in any way explain the specificity of normal antibodies. On the other hand, the enormous number of possible specificities which may be produced in antibody formation by an unlimited number of antigenic substances does not permit the acceptance of the "side-chain" view of Ehrlich. Landsteiner (34) sums up the question by saying that no other conclusion is possible except to regard the production of antibodies as a synthetic function of the animal body; and although we cannot understand the underlying mechanism, we must assume that by immunization the physiological processes are modified in a manner contingent upon the nature of the antigen, leading to the production of antibodies which are adapted as closely as possible to the immunizing antigen. Such a concept conforms to the observed fact that the properties of antibodies depend not only on the antigens but on the species and individuality of the immunized animals and the method used for immunization as well. We will see later that there is actually a demonstrable difference, both chemical and functional, between antibodies produced against the same antigen in rabbits on the one hand and in horses on the other. As far as actual chemical study is concerned, it has not been possible to separate specific antibodies from serum globulins. And all attempts to characterize antibodies as different from ordinary serum globulins have been unsuccessful except that diffusion experiments have suggested that antibody is a serum globulin of higher molecular weight than normal globulin, and Felton has shown his purified antipneumococcus euglobulin to be free from phosphorus and of an iso-electric point different from any other horse serum globulin. It is impossible, therefore, to make any more definite statement about antibody formation except that it may be considered as an alteration in the synthesis of serum protein, a mod-

ification being brought about by affinities for the antigens present in the cells.

Chemistry of Antibodies. We have already noted that all attempts to purify antibodies have led to the uniform conclusion that these immunologically active serum constituents are inseparably bound up with serum globulins. This is consistent with observations made as early as 1903 by Joachim (35) and variously confirmed by Ledingham (36) and others to the effect that during immunization there is a quantitative increase of globulins over those present in normal serum. In subsequent years the results of all investigators have not consistently confirmed this, but the bulk of evidence is in favor of such quantitative globulin increase. Quite recently the work of Boyd and Bernard (37) has strengthened this view in studies of rabbits immunized with ovalbumin showing at the same time that the almost invariable rise in globulins during immunization affects mostly the fractions precipitated by 13.5 and 17.4 per cent sodium sulfate.

It is quite apparent, however, that while antibodies undoubtedly are associated with the globulins, there is no regularity with which antibodies of different kinds are distributed in individual globulin fractions. There is also a difference in such distribution according to the species of animal in which immunization is practiced. Diphtheria antitoxin in the horse seems to be associated chiefly with pseudoglobulins, whereas in goats the same antibody, according to Banzhaf and Famulener (38), is chiefly found in the euglobins. In antipneumococcus serum of horses Felton (39, 40, 41, 42, 43) has shown that the protective substance is always present in the water-insoluble euglobulins and is precipitated by relatively low concentrations of ammonium sulphate.

The concentration of antibodies has been attempted since the early work of Gibson (44) and Banzhaf and Gibson (45) by various methods of salting out. The modifications of these methods at present being used are too many to be described except in principle. The purification of diphtheria antitoxin, for instance, by the original Banzhaf-Gibson process, consisted in a preliminary heating of the serum to 56° C. for several hours, by which, without loss of antitoxic value, some of the water-soluble or pseudoglobulin was converted into a state of insolubility approaching that of the euglobulins. Thirty per cent ammonium sulfate was then added, and the precipitate or euglobulin removed. In the filtrate which contains most of the antitoxin the ammonium sulfate concentration was now brought to 45 per cent, at which concentration most of the antitoxin comes down.

In pneumococcus serum the most common methods of concentration of antibodies at the present time are based upon Felton's studies, which have shown that in horse serum the protective substances are associated

with a water-insoluble euglobulin-like substance. In his original procedure Felton obtained most of the antipneumococcus protective bodies by merely diluting with about 20 volumes of water. Later he developed methods of precipitating with about 30 per cent saturation of ammonium sulfate and by sodium sulfate fractionation. Felton has also shown that practically all the protective substances present in horse antipneumococcus serum could be precipitated by 15 to 20 per cent ethyl alcohol at zero degrees. The precipitated protein contained both water-soluble and water-insoluble material but can be washed with water to remove the former and the latter, dissolved in salt solution, contains practically all the protective antibodies present in the original serum.

Lipoids and Antibodies. Efforts to define the chemical nature of antibodies have been made by a number of investigators by extracting lipoids from antisera such as rabbit anti-horse, horse antipneumococcus serum, etc. Hardy and Gardiner (69) found that extraction of diphtheria antitoxin by alcohol-ether in the cold, left behind a lipoid-free globulin which was soluble in salt solution without change of antitoxic properties. Hartley (70) used similar methods on a number of antisera and found a loss of precipitating potency for the lipoid-free antiprotein sera but no marked change in the antibody potencies of diphtheria antitoxin and antityphoid sera. Horsfall and Goodner (71) removed the lipoids from Type I antipneumococcus horse serum and found that such extraction brought about loss of agglutination and precipitation powers. The initial activity of the type-specific antibody, however, could be restored in the case of horse antisera by the addition of lecithin and in the case of rabbit serum by the addition of cephalin. The protective antibody of extracted horse serum was only partially absorbed by washed pneumococci, in contradistinction to the complete absorption of the protective antibody from unextracted horse serum. The significance of these investigations is not yet clear.

The Union of Antigen and Antibody. Since, as we shall see, antibodies are proteins — more precisely globulins — the studies of Loeb (46) are pertinent to the problems of antigen-antibody union.

Loeb's work indicated that proteins may be regarded as amphoteric electrolytes which can exist in three states, according to the hydrogen ion concentration. He showed that at the iso-electric point, protein exists in a practically non-ionized condition, and is able to form neither proteinate nor protein acid salt. On either side of the iso-electric point, however, the protein combines like any other chemical compound, with acids, salts, and perhaps other substances. Thus, in the case of gelatin, with its iso-electric point at *p*H 4.7, when the hydrogen ion concentration is on the acid side of this point, combination can take place only with the anion of an electrolyte, forming, let us say, gelatin chloride. When the *p*H is greater than 4.7, or on the alkaline side, it can unite with the cation only, forming sodium

gelatinate, etc. Loeb conceives the protein molecule as presenting various atom groups for combination with materials with which it comes in contact, which, on the alkaline side of the iso-electric point, act something like fatty acids which are able to form salts with metals, etc., perhaps by combination with the COOH groups of the molecule. On the acid side of the iso-electric point the reverse is true, the proteins reacting like ammonia and being capable of uniting with only the anion of electrolytes, perhaps through their NH_2 groupings.

That the principles set forth by Loeb have definite bearing on antigen-antibody union is apparent from the studies of Coulter. Coulter (47, 47 a) determined the movement of normal and sensitized red blood cells in an electric field and found that the movement of such cells in the field is a function of the hydrogen ion concentration. The iso-electric point for the cells was *p*H 4.6. On the alkaline side they carried a negative charge and on the acid side a positive one. There is an old observation by Joos (48) that the salt which is necessary for the agglutination of bacteria did not act indirectly as supposed by some, but combined chemically with the bacteria. Joos using the minimum amount of salt necessary, found that it disappeared from the supernatant fluid; but until very recently this observation was neither confirmed nor further pursued. Coulter reinvestigated this and found that, like protein, both normal and sensitized cells combined chemically with inorganic ions. By adding NaCl and HCl he found that the cells combined with the chlorine ions on the acid side of the iso-electric point, taking up amounts much greater than any they would take up on the alkaline side. On adding NaOH to such mixtures, he found that chlorine is given off by the cells. Conversely, when he suspended the cells in isotonic barium chloride solutions, the barium ion was absorbed on the alkaline side of the iso-electric point. This behavior corresponded to that found by Loeb for gelatin and other protein suspensions, which combined with the cation on the alkaline side of the iso-electric point, and the anion on the acid side. He found, further, that the optimum agglutination for normal cells is at *p*H 4.75, a point at which the cells supposedly carried no charge in relation to the surrounding medium, and are, in their most chemically pure form, uncombined with inorganic ions. The optimum for the agglutination of sensitized cells at *p*H 5.3 is probably related to the optimum for the flocculation of the immune bodies in the serum.

Of still greater significance are the experiments of Coulter on the equilibrium between sensitizer and red cells in relation to hydrogen ion concentration. Working in a salt-free medium, isotonic saccharose solution (9.2 per cent), he measured the proportionate amounts of sensitizer which combined with the cells at various hydrogen ion concentrations, and, conversely, the amount of sensitizer which dissociated from the saturated cells when they were brought into a similar range of hydrogen ion concentrations. Both measurements corresponded, showing that in the combination a definite equilibrium is maintained between the two for each *p*H. He found that the maximum combination between sensitizer and cells took place at or about *p*H 5.3, the approximate iso-electric point of the serum globulin in which the immune bodies are carried, according to Rona and Michaelis (49). At this point the combination between the two was almost 100 per cent. On both sides it diminished rapidly, on the alkaline side being reduced to not much more than 5 per cent at a *p*H of about 10. On

the acid side it also diminished, but could not be measured to the same degree because of the hemolytic action of the increasing acidity.

The addition of sodium chloride greatly increased the proportion of sensitizer combining with the cells at all reactions except those near the isoelectric point where the union between the two seemed independent of the salt.*

We may assume, therefore, that the maximum union is at a point where almost all the immune body is present as an undissociated molecule not in combination with inorganic ions. The dissociated ions of the sensitizer formed either by its acid or basic dissociations, do not appear to unite with the cells. The salt solution, again in conformity with Loeb's observation, seems to depress the ionization, and, therefore, render the combination relatively independent of the *p*H.†

This reversibility of the antigen-antibody reaction has been the subject of many investigations. Landsteiner (51) and Landsteiner and Jagic (52) in 1902 found that when red blood cells were agglutinated by abrin, the agglutinated cells then rapidly washed in cold salt solution and finally immersed in a salt solution at 42° C., a certain amount of the abrin was split off and could be recovered. Experiments with normal agglutinins showed the same thing, the most successful dissociation of agglutinins from agglutinated typhoid bacilli being obtained at 55° C. At about the same time, Morgenroth (53) showed that when red blood cells were sensitized with hemolysin or amboceptor, and were then brought into contact with unsensitized cells, some of the hemolytic antibody was dissociated from the sensitized cells and became attached to the freshly added unsensitized ones. Bail and Tsuda (54), Spaet (55), Hahn and Trommsdorf (56), Von Liebermann and Fenyvessy (57) digested sensitized pig corpuscles in N/100 hydrochloric acid in salt solution, precipitated the extracts with alkali, purified the precipitate with ether, and found that the final solution contained antibodies.

Kosakai (58) washed sensitized sheep cells in saccharose solutions, and succeeded in recovering about five sixths of the antibody combined with the antigen. Huntoon (59) worked chiefly with pneumococci. He developed a method by which he can dissociate a large proportion of the antibody from sensitized penumococci by washing the agglutinated pneumococci with 0.5 per cent sodium carbonate in salt solution. Such solutions could be repeatedly filtered without loss of antibody, and, therefore, obtained in a sterile manner.

Absorption to an antigen and subsequent dissociation has been widely used as a method for obtaining antibodies in as pure a state as possible. The report of Salkowski (60) that he had produced a protein-free diphtheria antitoxin, Heidelberger (61) regards as having been satisfactorily refuted by Dold and Freudenberg (62). Huntoon's (63) work on pneumococcus antibody has been mentioned above and has been extended

* The relationship of electrolysis to agglutination and precipitation phenomena is further discussed in the section on agglutination.

† For more complete discussion of these problems see Marrack (50). Also see chapter on Agglutination and Precipitation for discussion of union of antigen and antibody.

by Ottenberg and Stenbuck (64, 65, 66). Ottenberg and Stenbuck dissociated agglutinins from sensitized typhoid bacilli and precipitated them at *p*H 6.4 with slight concentrations of copper ion. There is no doubt that both Huntoon and Ottenberg obtained extraordinarily pure but not protein-free antibodies.

Locke and Hirsch (67) obtained hemolysin of great purity by absorbing fresh rabbit antiserum with washed sheep cells. The serum was used in active condition and the cells completely laked. The stroma was then centrifugalized and 90 to 99 per cent of the hemolysin became attached to the stroma sediment. This stroma was suspended in salt solution and repeatedly washed and centrifugalized until no trace of color remained. The hemolysin sticks to the stroma and this stroma-hemolysin was three times extracted with ether. The ether was removed by centrifugation in a warm centrifuge and the residue again washed. Considerable hemolysin is lost in this washing, but protein impurities are removed. The washed residue was extracted with N/1000 sulfuric acid and the extracts flocculated by neutralization. The precipitated material extracted with salt solution yields a highly pure hemolysin, the protein content of which, per unit of hemolysin, is approximately 0.000044 mg., which, according to Locke, represents a purification of 1 to 150,000.

Chow, Lee, and Wu (68) have prepared what they call immunologically pure antibody by precipitating antipneumococcus horse serum by successive additions of homologous polysaccharide solution. They recovered the antibody from the precipitates by suspending in water to which enough N/70 NaOH had been added to dissolve the protein. The solution was then brought to *p*H 9.5 with weak hydrochloric acid at which the precipitate once formed could no longer redissolve. The antibody recovered in this way possessed strong agglutinating and precipitating power, and its protective titer was higher than that of the original serum. Subjected to denaturation by acid at *p*H 2.5 and to trypsin digestion, the loss of immunological properties ran parallel with the chemical modifications of the protein.

Purification by Digestion. A method for the purification and concentration of antibodies, the description of which we take from the U. S. Patent Office, is that of Parfentjev (72), which depends upon the partial digestion of antiserum with enzymes. In the case of diphtheria antitoxin a suitable amount of the serum is diluted with three times its volume of salt solution. To this is added an amount of U. S. P. pepsin, equivalent to about 1 per cent of the total volume of liquid. The hydrogen ion concentration of the mixture is adjusted to a *p*H of 4.2 by adding "suitable acid or alkali." The mixture is digested for about two days at 37° C. The purpose of the procedure is to digest almost completely the valueless albumin fraction with substantially no destruction of the antibodies contained in the globulins.

The digested material is filtered through suitable ceramic cones coated with collodium. By such ultrafiltration the digested material passes

through and the residue in the filters represents a purified concentrated antibody solution. Modifications of this method are being experimented with both by Parfentjev and others.

Nature of Antigen-Antibody Union.* In the union of antigen and antibody the very fact of specificity indicates that delicate chemical affinities must play a role. The chemical nature of the haptene group determines specificity and in the chapter on Antigens we have seen that the specificity of the union may be determined by a relatively small part of the antibody molecule. A consideration of what has been said about the determination of modified specificities in compound antigens will make this plain. Just how the union takes place is not entirely clear. It has been suggested (we refer the reader to the discussion in the chapter on Agglutination and Precipitation) that the antibody may be present on the antigen as a film of specifically adsorbed protein and that in highly dispersed antigens, as in precipitin reactions, the antibody globulin represents the bulk of the material of the precipitate. At the same time the antibody globulin becomes denaturized, that is, becomes relatively water insoluble. Eagle suggests that the specificity of the antibody molecule is determined by its hydrophilic groups and that the so-called denaturization means that now, the hydrophilic groups being attached to the antigen, the hydrophobic ones face the water phase. Marrack objects to the film hypothesis largely on the basis of the quantitative studies of Heidelberger and Kendall, which showed, for instance, that at equilibrium 0.5 mg. of pneumococcus carbohydrate may combine with 27 mg. of the immune globulin. This would mean a globulin layer of up to 27 Ä and that consequently one must assume that in certain unions the antibody molecule combines not as a film but as a closely packed group of globulin molecules.

The quantitative relationships of antigen and antibody combinations have been much clarified by the methods of Heidelberger and Kendall (73, 74, 75), who worked with Type III Pneumococcus antibody from horse serum and a chemically pure Type III soluble specific carbohydrate. The antibody was purified by one of Felton's methods in which the serum was mixed with ice-water, adjusted with phosphate buffers to a *p*H range of 3.6 to 6.3. The soluble specific Type III substance was ash-free, containing 0.04 per cent of nitrogen. They expressed quantities of antibody in the precipitates as nitrogen multiplied by the factor 6.25. When increasing amounts of the carbohydrate were added gradually to constant amounts of antibody, a point was first reached at which only slight traces of antibody were left in solution. Further addition brought about a state in which either both carbohydrate and antibody were present in traces, or neither could be detected.

* See also chapter on Toxin-Antitoxin Reactions

This they assumed to be about the equivalent point. Then more carbohydrate was added until a slight trace of free S III was present. The entire range of such mixtures they called the "equivalence zone," assuming that the midpoint represented practically equivalent union. In their early experiments they found that the ratio of the two components within this range changed from approximately 120–1 at the point of the smallest amount of determinable precipitate to about 60–1 at the point at which both reagents could be detected in the supernatant fluid. When, in later experiments, the mixtures were carried out exactly as described above, they found a much wider range within the "equivalence zone." In the region of slight antibody excess, antibody was to polysaccharide as 40–1, whereas in that of slight excess of the polysaccharide, the relationship was less than 5–1. There was thus a tenfold variation within the quantitative range in which antibody and carbohydrate could combine. Moreover, it was found that the amount of antibody nitrogen precipitated under any given conditions was independent of actual antibody concentration, but dependent upon the relative proportions of antibody and polysaccharide.

From these results they conclude — and it seems to us on valid grounds — that the two reagents are multivalent in regard to each other. These matters are discussed in more detail in the chapter on precipitation.

Differences in Antibodies in Animal Species. Considerable differences have been found to exist between anti-serum produced in rabbits and that obtained from horses. The most important of these were the following:

Unconcentrated rabbit antiserum has a much higher mouse protective potency than the horse antiserum.

The rabbit antibody, as demonstrated by ultrafiltration (76) and ultracentrifugation (77), is apparently a much smaller size (molecular weight of 150,000) than the horse antibody.

Rabbit antiserum does not show the prozone phenomenon which is so prominent in the case of horse serum that greater than the optimum amounts give no mouse protection whatever.

According to Goodner and Horsfall (78), the mouse protective action of horse antipneumococcus serum is inhibited by cholesterol and cephalin. These lipoids do not alter the protective action of rabbit antiserum.

Furthermore, the rise of antibodies is much more rapid in the immunization of rabbits than it is in the case of horse immunization.

It is quite apparent that antibody formation in different animals is different both qualitatively and quantitatively, and that chemical and physical properties in the several animal species lend these antibodies properties which are of great immunological significance. Size and distribution between euglobulins and pseudoglobulins are so far the

only demonstrable facts from which we can reason. Whether or not more precise chemico-physical definition will be possible is a question of the future.

On the Essential Identity of the Antibodies (79, 80). Ehrlich's (81) first analysis of antibodies postulated the conception that agglutinins, precipitins, sensitizers, bacteriolysins, hemolysins, or the so-called amboceptors, opsonins, and the anaphylactic antibodies were separate substances formed in the animal body in response to treatment with a single antigen. Kraus (82) in the first edition of Kolle and Wassermann's Handbook, summarizes this point of view in the following words:

"Just as the bacterial body contains a variety of different antigens, so we may assume that animal protein is made up of a large number of different antigenic elements. If the animal body is treated with such substances and finds corresponding receptors, there results the formation of a variety of qualitatively different antibodies. . . ."

When Gengou (83) in 1902 noted that alexin or complement was fixed when a precipitating antiserum was added to its homologous antigen, he interpreted this as meaning that, in addition to precipitins, the antiserum contained other antibodies, the "albuminolysins." To be sure, Gay and Moreschi (84, 85) showed that the fixation of alexin was chiefly a property of the precipitate which was formed, but this was regarded as signifying that the protein sensitizers were mechanically carried down during the precipitation.

In the course of the subsequent years, however, many facts turned up which suggested the possibility that the different manifestations of antibody reaction might be explained by a single immune substance active under a variety of environmental conditions.

The idea that agglutinins and precipitins might represent one and the same antibody has been more or less prevalent from the beginning. It originated with Paltauf (86) and was expressed by v. Eisler (87).

Bail and Hoke (88), who studied the bacteriolytic, precipitating, and agglutinating actions of normal beef serum and immune rabbit serum upon cholera spirilla, expressed the opinion that there were no separate bacteriolytic, precipitating, or agglutinating antibodies in these sera; that the essential fact was the existence, in the sera exerting these effects, of a single antibody, and that the various reactions which followed were the results of the conditions under which the different observations were made. Although a similar idea, on less valid evidence, had been expressed by Bürgi (89), and although Bail and Tsuda (54) followed out the thought in subsequent publications, the view made little headway.

In order that there may be no ambiguity as to just what is meant by what we call the "unitarian" view, let us formulate it clearly.

By such a conception it is not implied that a complex cell like, for instance, the typhoid bacillus gives rise to one variety of antibody only. There may be formed a specific sensitizing antibody against the major chemical constituent, and other sensitizers against other antigenic substances enclosed in the same cell body or contained in the same antigenic solution. But we do mean that, were we working with a single antigen, in a pure state, one variety of antibody only would be produced. This would be present in the form of a serum constituent specifically capable of uniting with the antigen. As a result of the union, the antigen is altered in its physical and in its chemical behavior. The resultant reactions which may be observed with this sensitized antigen (agglutination, precipitation, complement fixation, bactericidal phenomena, bacteriolysis, opsonization, or sensitizing effects in the anaphylactic sense) are determined not by differences in the nature of the antibodies with which the antigen has united, but rather by the physical state of the antigen itself, the nature of the co-operative substances (alexin, leucocytes, tissue cells), and by the environmental conditions under which the observations are made.

Thus, if the antibody comes in contact with a very finely divided antigen, as in a bacterial extract or in, let us say, horse serum, if electrolytes are present, precipitation occurs.

When we are dealing with whole bacteria of relatively large mass and correspondingly small surface exposure, agglutination is the result, and quantitative parallelism with the precipitin reaction is not to be expected because of the much greater dispersion of the antigen in the latter test.

When alexin is present, complement fixation or hemolysis or bactericidal effects result, since the changes produced by the sensitization have rendered union with the complement possible.

When there are leucocytes present, the union makes possible the phagocytosis of the antigen; and when the antibody is absorbed by the cells of an animal, anaphylactic "sensitization" occurs.

Earlier opposition to such a view was largely based upon lack of quantitative parallelism between agglutination and precipitation curves on the one hand and bactericidal or protective antibody curves on the other.

In appraising such objections, however, it must not be forgotten that agglutination and precipitation are actually secondary phenomena, after the union of antigen and antibody has taken place, and are dependent upon environmental factors.

Heidelberger and Kabat (90) have been able to place the identity of agglutinin and precipitin upon an exact quantitative basis. Working with Type I antipneumococcus horse serum and type-specific polysaccharides, they found that agglutinating and precipitating substances are present in identical amounts in the serum and that a reduction in one is

accompanied by a quantitatively identical reduction in the other. The quantitative problems involved in this question are dealt with in the section on precipitins.

In 1912 Dean, analyzing the relationship between alexin fixation and precipitation, concluded that the proportions of antigen and antibody which favor rapid and complete precipitation do not favor complete alexin fixation. He did not believe that the two reactions followed a parallel course, but said that he thought they represented two phases of the same reaction, a "flocculation representing the first stage of a change that can be recognized in its early stage by complement fixation." In 1912 we were engaged in a similar analysis from which we came to the conclusion that there was no need for assuming that the antibodies which were involved in the fixation of the alexin were essentially different from those that brought about the precipitation. In his Croonian lecture of 1917, Dean independently came to the same conclusion that we did, namely, that all the various so-called antibody reactions were attributable to one and the same specific substance in the serum.

That the anaphylactic passive sensitizing effect of sera was found to be quantitatively proportionate to the precipitin contents of these sera, was shown by Doerr and Russ (91) in experiments which seem completely to justify the identity of precipitating and anaphylactic antibodies suggested by Friedberger in 1908.

Observations made by Coulter (47) have bearing upon this question in indicating that agglutination and sensitization to hemolysis of red cells are both due to the action of one substance. Coulter found that "the optimum hydrogen ion concentration for the agglutination of sensitized cells (rabbit-antisheep sensitizer), in a salt-free medium, occurs at *p*H 5.3, which corresponds with the optimal point for the precipitation of the serum-globulin itself."[1] The optimum for precipitation of serum-globulin, in which the immune bodies are carried, is stated by Rona and Michaelis (49) as *p*H 5.2 and the iso-electric point for typhoid immune bodies as *p*H 5.4. This reaction is more alkaline than that which is optimal for the agglutination of normal sheep cells in saccharose solution, so that Coulter concludes, as far as agglutination is concerned, the behavior of sensitized cells is closely related to the properties of the immune globulin.

These observations indicate that the hydrogen ion concentration at which the agglutination of sensitized cells is most complete corresponds with the iso-electric point of that part of the serum which contains the antibodies and also corresponds with the point at which the largest amount of hemolytic sensitizer is absorbed by the red cells. This constitutes strong evidence in favor of identity of the hemolytic sensitizer and the agglutinin.

Final confirmation of the unitarian theory has come from the quantitative precipitin studies of Heidelberger and from work with immunologically pure precipitin of rabbit origin such as that of Chow and Wu (92).

On the basis of the unitarian theory of antibodies, then, we may summarize antigen-antibody reactions in general in the following manner:

The specific stage of the union is the combination of the antigen with the antibody globulin. This is due to affinity between what Marrack calls the "determinant" group of the antigen and the immune globulin. The nature of the determinant groups has been discussed by Marrack (50), and has been to some extent elucidated by the studies of Landsteiner and others on compound antigens. As a result of the union the antigen is now coated by a layer of the specific globulin which, in the opinion of Eagles, possesses the properties of denatured protein. Marrack takes the view, on the other hand, that instead of representing a surface film, the antibody molecules are attached to the antigen in such a manner that the polar groups on which the solubility of the globulin normally depends "are brought into apposition with each other and attract each other instead of water molecules." Whatever may be the principles by which the union takes place, the antigen is now so changed both in regard to chemical and physical state that it becomes susceptible to alexin action (bactericidal effect), to engulfment by leucocytes (phagocytosis), and to flocculation in the presence of electrolytes (agglutination, precipitation). These effects represent the second stage of the reaction and are subject to environmental conditions which will be considered in connection with the several antibody reactions.

The Zone Phenomenon in Antigen-Antibody Reactions. When antibodies are titrated against antigens, whichever one is kept constant, there is a peculiar phenomenon which indicates that reactions are either slowed down or inhibited by an excess of one or the other of the reacting ingredients. With a constant antigen, excess of antibody produces inhibition only when the excess becomes a great one. An excessive antigen, on the other hand, always inhibits serum reactions.

The older interpretation of such zones have all been abandoned in view of the more thorough understanding of the nature of antigen-antibody reactions. Marrack (50) has discussed the matter from the point of view of the formation of the union of the two substances by a packing of antibody molecules around those of the antigen. Streng and Shibley in their studies on agglutination have shown that prozones of sera have been greatly increased by moderate heating. Marrack's interpretation is that the occurrence of the prozone in these heated sera is attributable to the presence of modified antibody which is preferentially absorbed by antigen but does not produce agglutination, acting, therefore, like Ehrlich's

agglutinoids. Such modified antibody can be removed either by filtering through a Berkefeld "N" candle or adsorbed with talc. Presumably, as Marrack states, as the result of partial heat denaturation of antibody protein, a partial loss of affinity for water has been produced. This accounts for its ready absorption by a filter and would also render likely the preferential absorption by the antigen, providing it was undamaged in its specific binding power. While this is not the whole explanation, the presence of prozones seems at least in part to be due to the presence of modified antibodies in the sense just discussed.

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CHAPTER VI

TOXIN-ANTITOXIN REACTIONS

WHEN Behring and his collaborators, Kitasato and Wernicke, had shown that the cell-free blood serum of animals immunized with tetanus and diphtheria toxins respectively possessed the power to protect other animals against the poisons, it became important to determine the mechanism by which the "antitoxic" effect was attained. The earlier opinion, expressed by Behring himself, held that the toxin was injured or destroyed by the antitoxic serum. Doubt was thrown upon this explanation by the experiments of Roux and Vaillard (1) and by those of Buchner (2). The work of the former showed that mixtures of tetanus toxin and antitoxin, measured in such proportions that they were harmless for normal guinea pigs, could still be found toxic for animals weakened by preliminary inoculation with other bacteria. Buchner found that similar mixtures, harmless for mice, were still toxic for guinea pigs. He inferred from this that cell reactions influenced the antitoxic effect. This conception implied an indirect protective function on the part of the antitoxin, not due to direct reaction between it and the poison. That this explanation, too, was faulty was first made evident by studies on snake venoms.

In 1894 Calmette (3) and Physalix and Bertrand (4) independently succeeded in obtaining an antitoxin against snake poison. Calmette (5) determined that the venoms of the naja and cobra remained potent after short exposures to 100° C. The antitoxins to these poisons were destroyed at much lower temperatures. When mixtures of the two substances, so proportioned that their injection into animals was innocuous, were heated to 68° C., toxic properties again became evident, a demonstration that the toxin had not been destroyed, but had remained neutral only in the presence of the intact antitoxins. These experiments were confirmed by Wassermann (6) for pyocyaneus toxin and antitoxin.

In the experiments on snake venom, Calmette interpreted the restitution of toxicity after the heating of neutral mixtures of cobra neurotoxin and its antitoxin as evidence "qu'il ne s'était pas formé aucune combinaison de ces deux substances ou que la combinaison réalisée était, au moins, très instable." Morgenroth (7) examined these relations and found that the addition of a small amount of hydrochloric acid to mix-

tures of snake poison and its antitoxin resulted in the dissociation of their union. To mixtures of the venom and its antitoxin, neutralized and even overneutralized, he added hydrochloric acid until the total concentration amounted to N/18. By this method a toxin-HCl modification was produced which was dissociated from its union with the antitoxin and was extremely resistant to heat. In such a mixture of toxin and antitoxin, heating at 100° C. in a water bath for 30 minutes destroyed the thermolabile antitoxin and undiminished toxic properties could be demonstrated.

These researches form confirmation of the original experiments of Calmette and prove that the inhibition of harmful properties of any true toxin, after mixture with its antitoxin, does not depend upon toxin destruction. But while Calmette interpreted the facts as pointing toward a failure of union of the two substances, Morgenroth's work is not incompatible with the conception of a neutralization.

The nature of such neutralization was then systematically studied by Ehrlich with ricin and antiricin. It had been shown by Kobert and Stillmarck that ricin (the powerfully poisonous principle of *Ricinus communis*, castor oil bean) would agglutinate the red blood cells of a number of animals. Ehrlich recognized how closely analogous the neutralization of ricin by antiricin was to that of diphtheria toxin by its antitoxin. The reaction furnished a simple method of test tube experimentation since the agglutinating effects of ricin upon rabbits' corpuscles could be inhibited by the addition of antiricin. A visible reaction was thus available, which excluded the participation of the tissue cells.

By means of this method Ehrlich (8) found that neutralization was accelerated by moderate heat and by concentration of the reagents and that the reaction followed roughly the law of direct chemical reactions in general. When he added 0.3, 0.5, 0.75, 1.0, etc., cubic centimeters of serum from a ricin-immune goat to constant quantities of ricin, and then added rabbit cells, the hemagglutinating properties of the ricin were inhibited in direct proportion to the amount of antiricin mixed with it. His test tube experiments moreover represented the occurrences within the animal body, since similar mixtures injected into mice were toxic in direct proportion to the balance of ricin and antiricin established in the injected material.

The views of Ehrlich and his followers formed the starting point for efforts to establish a standard of strength for diphtheria antitoxin.

Antitoxin Standardization. Earlier attempts to standardize diphtheria antitoxin by the use of living cultures (Roux and Behring) were abandoned, since it was found that accurate establishment of fixed lethal doses of the culture was not possible. When the facts, just recorded, concerning the quantitative relations of the soluble toxins and their

respective antitoxins came to light, Behring attempted the standardization of curative sera by the use of toxins, both in tetanus and in diphtheria. As a base line he established for diphtheria poison an arbitrary toxin unit which he defined as the amount of any given diphtheria filtrate sufficient to cause death in a guinea pig of 250 grams, and, borrowing the terms from chemical nomenclature, he designated as a "normal" poison solution one which contained 100 such units in one cubic centimeter. (D T N, M250 = diphtheria toxin normal, Meerschweinchen 250 grams.)

Together with Ehrlich, Behring then established an antitoxin unit (I-E, Immunitäts Einheit). They designated as a "normal" antitoxic serum one "which contained in one cubic centimeter one antitoxic unit" (I-E), and state further, "of this serum 0.1 cc. neutralizes 1 cc. of the Behring normal toxin."

Subsequent experience, however, soon demonstrated that such simple titration of a "normal" toxin solution against a "normal" antitoxin was quite impracticable. Toxin, as we shall see presently, is quite unstable, and the relationship, during deterioration, between toxicity and capacity for union with antitoxin is not a regular one. This will become obvious as we proceed. At any rate, in measuring the quantitative relations between two substances which were titrated only by their effects upon each other in the animal experiment, it became necessary to set up an arbitrary base line for one of them. This Ehrlich did by designating as one unit of antitoxin the amount of a relatively potent serum, then in his possession, which neutralized one cubic centimeter of Behring's "normal toxin." This serum he preserved from deterioration by drying and, with it, he began to make the measurements on which our present conception of the "antitoxin unit" is based. An actual definition of this unit, as at present in use, can be given only after discussion of the experimental procedure by which it is determined.

As the standard test animal guinea pigs of 250 grams were chosen, and improvements in the methods of measurement were introduced, in that the toxin and antitoxin, instead of being separately injected as heretofore, were mixed, allowed to stand for 15 to 30 minutes, and then injected together subcutaneously.

By means of this technique Ehrlich set out to examine a large number of toxins against his arbitrary antitoxin unit and obtained results which, aside from their practical value, have had an important influence upon the development of the knowledge of antigen-antibody reactions. These investigations were considerably complicated by the fact that neither the diphtheria toxin nor the antitoxin is very stable, and deterioration occurs unless special methods of preservation are employed. Since the antitoxin, however, is much less unstable than the toxin, the former is employed in order to preserve the standard, and is preserved in sealed U tubes with anhydrous phosphoric acid. Kept in this way, in black, light-proof boxes, and at low temperature, it may be preserved for years without appreciable

loss of value and may be renewed by accurate comparative measurements from time to time.

Ehrlich measured, in a number of toxic filtrates, the minimal lethal dose for guinea pigs of 250 grams, establishing a time limit for death in order to obtain more accurate comparisons. He designated as the new M.L.D. or "T" (that is, toxic unit) the quantity of toxin which will kill a guinea pig of the designated weight in from 4 to 5 days. He then determined for a number of poisons the exact quantity just neutralized by one antitoxin unit, calling this quantity L_0. (L meaning Limes or threshold.)

It is clear that in judging complete neutralization of a quantity of toxin by antitoxin, there may be a strong subjective element, since any very slight excess of toxin may cause unimportant local reactions such as edema or small hemorrhages, which could escape the attention of one observer while being noticed and recorded by another. In order, therefore, to exclude definitely all subjective features, Ehrlich now established another toxin value — "Limes death," now, for convenience, written L_+ — which eliminated all possible variations of personal perception. He designated by this symbol that quantity of toxin which not only neutralized one antitoxin unit but included enough toxin, in excess of this, to give the result of one free toxin unit, that is, to cause death in 4 to 5 days in a guinea pig of 250 grams. Since the three values just defined form the basis of Ehrlich's experiments as well as that of all practical diphtheria serum standardizations we will briefly restate them for the sake of clearness.

Thus:

M.L.D. or "T" = the amount of toxin which, subcutaneously injected, causes death in a 250-gram guinea pig in from 4 to 5 days.

L_0 = the amount of toxin which is just neutralized by one antitoxin unit so that no trace of reaction, local or otherwise, ensues

and

L_+ = that amount of toxin which will cause death in 4 to 5 days in a guinea pig of 250 grams if injected together with one antitoxin unit.

It will further clarify the meaning of these terms to examine experimental protocols which show how these values are determined.

Thus in the following:

I. Injections of toxin
- (1) .005 cc. — guinea pig lives.
- (2) .009 cc. — guinea pig dies in 6 days.
- (3) .01 cc. — guinea pig dies in 4 days.
- (4) .02 cc. — guinea pig dies in 2 days.

.01 = M.L.D. or T.

II.* 1 Antitoxin unit + .14 cc. toxin = no reaction.
1 Antitoxin unit + .15 cc. toxin = no reaction.
1 Antitoxin unit + .16 cc. toxin = slight congestion about point of injection, scarcely visible.
1 Antitoxin unit + .17 cc. toxin = apparent reaction at site.
1 Antitoxin unit + .18 cc. toxin = edema at site.
L_0 = .16.

* II and III are taken from the article by Rosenau (9).

III. 1 Antitoxin unit + .19 toxin = late paralysis.
1 Antitoxin unit + .20 toxin = sometimes late paralysis and sometimes acute death.
1 Antitoxin unit + .21 toxin = death fourth day.
1 Antitoxin unit + .22 toxin = death in 2 to 3 days.
.21 = L_+ dose.

In determining these values with a large number of toxins Ehrlich discovered the curious fact that, although there was a rapid and extensive diminution of toxicity in every toxic filtrate in the course of time, there was no corresponding alteration in the L_0 amount. In other words, although more and more of the toxic broth was necessary to kill a guinea pig of standard weight in the required time, the amount of the same broth which neutralized one antitoxin unit remained approximately the same.

In seeking an explanation for this apparent paradox, Ehrlich concluded that we must assume that the toxin is complexly constructed, consisting of a toxophore and a haptophore group. Assuming that chemical union between the toxin and the antitoxin (or, in disease, the body cell) takes place, it is by means of the haptophore group that such union is brought about. The toxophore group, however, is the element by which toxic action is exerted after union by the haptophore group has been accomplished. It would be conceivable, therefore, that in deteriorating in toxicity the toxin might undergo alterations in the toxophore group only, its haptophore group, and, therefore, its antitoxin neutralizing properties remaining intact. Such modified toxins Ehrlich referred to as "toxoids" and, as produced by the action of formaldehyde, at present are spoken of as "anatoxins."

In the production of diphtheria toxins for practical purposes it has been found advisable to allow them to "season," that is, to stand for prolonged periods until they have reached a state of "equilibrium" at which the conversion of toxin to toxoids has been reduced to a minimum and the change of relationship between L_0 and "T" or M.L.D. has practically ceased to go on. From the very beginning of the growth of the culture in the incubator toxoid formation has probably occurred, and even freshly prepared toxic filtrates therefore are not pure "toxins."

In spite of the presence of such alteration products, in comparing the values L_0 and L_+ of any given toxin preparation, one would naturally suppose that L_+ minus L_0 should be equal to one M.L.D., or the quantity just sufficient to kill a guinea pig of 250 grams in 4 to 5 days. For we have seen that L_0 just neutralizes one antitoxin unit, while L_+ is the quantity which, in addition to such neutralizing power, has an excess of toxin equal in action to one minimal lethal dose. This, however, is not the case. Let us illustrate this by a concrete case. One of Ehrlich's

toxins on measurement showed a minimal lethal dose or M.L.D. of 0.0025 cc.

The L_+ dose of this was .25
while The L_0 dose of this was .125
The difference was .125, or 50 M.L.D. instead of 1 M.L.D. as we would suppose.

Stated in words, this measurement means that after neutralizing completely one antitoxin unit with the toxic filtrates, in order to obtain death in a guinea pig in 4 days with such a mixture, it was necessary to add, beyond the neutralizing quantity, 50 M.L.D., or again as much as was necessary for neutralization.

This last relation is merely coincidence, since it might have been 30 or 40 or 60 M.L.D. just as well. The important point is the fact that more than 1 M.L.D. was necessary, and by this fact Ehrlich was led to resort to an assumption which forms one of the basic principles of many of his explanations for serum phenomena, namely, the assumption of differences in combining avidity or affinity.

As applied to the present problem he reasoned as follows:

It is conceivable that the toxoids resulting from deterioration of toxin might possess three different degrees of affinity for the antitoxin. They might have a stronger, an equal, or a lesser affinity than the toxin itself. If their affinity for antitoxin were equal to that of toxin they would, of course, not influence the L_+ dose itself; if stronger than toxin their influence would be so exerted that toxin would be forced out of combination with antitoxin, giving place to the toxoid, and the effect would be the opposite from that experimentally observed. If, however, their affinity for antitoxin were weaker than that of toxin, each additional toxin unit added to the L_0 dose would unite with antitoxin, replacing a corresponding quantity of the toxoid of weaker affinity. In consequence, as far as the poisonous properties of the mixture are concerned, the addition of toxin would not render the neutral mixture poisonous for guinea pigs until the toxoids had been completely displaced from union with antitoxin. Finally, after all the antitoxin had been bound to unchanged toxin, further addition would result in the presence of free toxin and poisonous properties would again appear in the mixture, as in the following illustration.

151 toxin-antitoxin + 49 epitoxoid-antitoxin = L_0.
Add 1 M.L.D or T and we have:
152 toxin-antitoxin + 48 epitoxoid-antitoxin + 1 epitoxoid free.
Add 2 M.L.D or T and we have:
153 toxin-antitoxin + 47 epitoxoid-antitoxin + 2 epitoxoid free until, finally, adding 50 T, we get:
200 toxin-antitoxin + 49 epitoxoid free + 1 toxin free = L_+.

In subsequent quantitative studies, Ehrlich reversed the procedure outlined above by making serial mixtures, each of which contained a constant L_+ amount of toxin with increasing fractions of an antitoxin unit. For each mixture, he then determined, by guinea pig injection, the amount of toxin neutralized or left free. Again, he encountered irregularities in the neutralization curves which led him to extend his "varying avidity" theory to the assumption that toxin solutions contained mixtures of toxoids of many different degrees of combining "affinities" (Affinitäten) for antitoxin. One outcome of this conception was his "toxin spectra" in which the composition of a given toxin solution in toxin and the various toxoids, was graphically presented.

Ehrlich's experimental facts contributed materially to the development of the subject. His conclusions, however, were soon challenged, most effectively by Arrhenius and his collaborators.

The views of Ehrlich were founded on the conception that the reactions between antigen (toxin) and antibody (antitoxin) took place in the manner in which highly ionized "strong" acids reacted with strong bases. The result would thus be one at which, at equilibrium in relatively dilute solutions, practically neither of the reacting substances would be present in the free, uncombined, and functionally active condition. Arrhenius and Madsen (10, 11) who may be credited with the first introduction of the methods of physical chemistry into the study of immunity, objected to this view on the grounds that the phenomena observed by Ehrlich could be explained much more simply by assuming that the toxin-antitoxin reaction was, both in regard to velocity and reversibility, similar to that taking place between a weak acid — for instance, boric or acetic acid — and a base like ammonia. In such cases the neutralizations would take place in a manner such that, at various equilibria, other things being equal, there would be present different amounts of combined and uncombined materials — calculable, in simpler systems, by the laws of Mass Action. The toxin-antitoxin reaction could thus be conceived as follows:

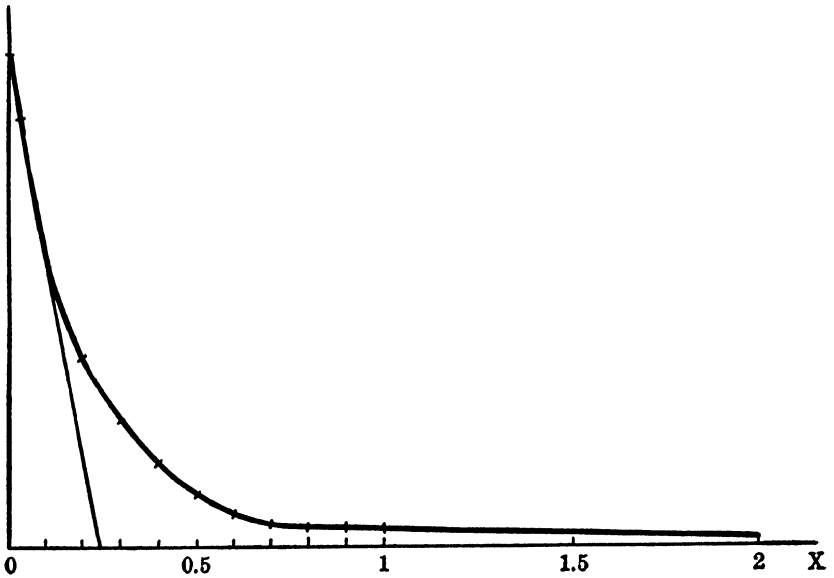
$$\frac{(\text{Free toxin}) \times (\text{Free antitoxin})}{(\text{Toxin-Antitoxin})} = K$$

or

$$(\text{Free toxin})\ (\text{Free antitoxin}) = K\ (\text{Combined toxin-antitoxin})$$

In support of this conception Arrhenius, utilizing the hemolytic action of ammonia on red corpuscles, showed that the neutralization of this hemolysis by boric acid follows curves quite comparable to those observed in the neutralization of toxin by antitoxin. Observed hemolytic toxicity of the mixtures followed closely the calculable concentrations of free ammonia. In comparing such ammonia-boric acid curves to neu-

tralization curves of tetanolysin by tetanus antitoxin, Madsen found a degree of similarity too close to be interpreted as experimental error or as chance.



CURVE REPRESENTING THE NEUTRALIZATION OF TETANOLYSIN BY DIFFERENT QUANTITIES OF ANTITOXIN

(Taken from Arrhenius, "Immunochemistry")

TOXICITY (Q) OF 0.1 N. NH_3 (1 EQUIVALENT) WITHIN EQUIVALENTS OF BORIC ACID

N = EQUIVALENTS OF BORIC ACID ADDED	QUANTITY OF FREE AMMONIA — *i.e.*, TOXICITY — OBSERVED	Q = AMMONIA TOXICITY CALCULATED FROM FORMULA	ΔQ OBS.
0	100	(100)	
0.17	85	79	15
0.33	69	64	16
0.67	43	42	26 : 2 = 13
1	25	27	18 : 2 = 9
1.33	20	18	5 : 2 }
1.67	13	13	7 : 2 } 2.5
2	10	10	3 : 2 }

(Taken from Arrhenius (13))

The basic conception of Arrhenius and Madsen that antigen-antibody union is, like other chemical reactions, reversible and comes to an equilibrium depending on the relative masses involved has been upheld by subsequent studies. But more accurate description of the secondary factors which govern the quantitative relations in the immune reactions

has been made possible by the relative purification of the reagents and by the more accurate development of the technique of flocculation phenomena.* For discussion of these problems in relation to antigen-antibody reactions in general we may refer to the work of Heidelberger and Kendall described in the chapter on Antibodies.

In regard to toxin and antitoxin Ramon's flocculation method and the preparation of toxin of 70 to 90 per cent purity by methods of fractional precipitation such as those of Eaton and of Pappenheimer (see chapter on Toxins) have made possible a degree of precision not formerly attainable.

Healey and Pinfield (14) worked by mixing toxin and antitoxin (units determined by flocculation method) in various proportions, shaking the mixtures and, after incubation at 40° C. for 10 hours, followed by two days at room temperature, filtering through Whatman No. 5 paper. The uncombined toxin or antitoxin in the filtrates was then determined by flocculation. The most important result obtained was the determination that a neutral mixture containing equivalent amounts of toxin and antitoxin (TA) will readily combine with one further added equivalent of antitoxin (TA_2). Toxin and antitoxin can, therefore, like the Pneumococcus III SSS and its antibody, in Heidelberger's and Kendall's experiments combine with each other in multiple proportions.

The work of Pappenheimer and Robinson (15) confirms this view and carries the analysis several steps further. They worked with highly purified toxin preparations and a pooled pseudoglobulin preparation of antitoxin. The toxin was diluted so as to contain exactly 300 L_f † and the antitoxin so that it contained 300 flocculating units per cubic centimeter. Antitoxin in 1 cc. amounts was placed in a series of test tubes and toxin added in increasing amounts in volumes of 4 cc. Nitrogen determinations were made on both reagents and on the washed precipitates that came down in the tubes. The method is like that of Heidelberger and Kendall in the work referred to above, and the results indicated that, as in the pneumococcus precipitin studies, there is in toxin-antitoxin flocculation an equivalence "zone" rather than an equivalence "point."

The table on the following page is taken directly from Pappenheimer and Robinson's paper.

Examination of this table and disregard of the minute traces of toxin and antitoxin excesses detectable only by intracutaneous rabbit inoculation, shows that a neutral or equivalence zone extended, for 300 units of antitoxin, from between 150 to 425 L_f units of toxin. So far these results again demonstrate that toxin and antitoxin can unite in more than one,

* The technique of flocculation is described in the chapter on Diphtheria.

† For definitions of these values see chapter on Diphtheria, where methods of titration are described.

possibly in at least three, proportions. Survey of the nitrogen determinations in the table shows with extraordinary consistency that the antitoxin nitrogen in the precipitates remains practically constant throughout and that the increase of precipitated nitrogen through the range of the "equivalence zone" is directly proportionate to the number of L_f units of toxin in the mixtures — taking 0.00046 mg. nitrogen per L_f. The ratio of antitoxin nitrogen to toxin nitrogen — through the "equivalence zone" — indicating the quantitative combining relationships, was from 7 at the limit of antitoxin excess, to 3.5 at the flocculation point and 2.5 at the point of toxin excess.

TABLE 1

A — Antitoxin 621 — 300 Units and 8.11 Mg. Nitrogen per Cubic Centimeter. T — Toxin GH3-4 — 0.00055 Mg. Nitrogen per Lf Unit

I	II	III Supernatant § ¶		IV	V	VI **
Units Toxin	Toxin * Nitrogen	Toxin Total Lf	Antitoxin Total Units	Nitrogen || in Precipitate	Antitoxin Nitrogen in Precipitate (IV − II)	Ratio A-Nitrogen / T-Nitrogen (V ÷ II)
	Mg.			*Mg.*		
50 †	0.023		190 §	0	0	
100 †	0.046		95 §	0	0	
150 ‡	0.069			0.386	(0.474)	(6.9)
175	0.081		Trace ¶	0.554	0.473	5.8
200	0.092			0.564	0.472	5.1
225	0.103			0.579	0.476	4.6
300	0.138			0.612	0.474	3.4
400	0.184	Trace ¶		0.661	0.477	2.6
425						(2.4)
450	0.207	Trace ¶		0.652		
500 ‡	0.230	100 §		0.359		
600 †	0.276	240 §		0	0	

* The nitrogen (column IV) precipitated by 200 Lf of toxin subtracted from that precipitated by 400 Lf and divided by 200 gives 0.00048 mg. nitrogen per Lf of toxin. The figure used in column II, however, is 0.00046 mg. nitrogen per Lf, the average obtained from six titrations including the above.

† No flocculation.

‡ Incomplete flocculation.

§ Determined by flocculation.

¶ By intracutaneous rabbit test.

|| Average of duplicates.

** Figures in parentheses were calculated for the ends of the neutral zone, assuming 150 Lf and 425 Lf and complete flocculation.

In their bearing on Ehrlich's views, previously discussed, these results are extremely interesting in their refutal of the theory of "avidities" to explain the discrepancy between L_0 and L_+ doses. Flocculation shows toxoid as well as toxin. And in the experiments of Pappenheimer and Robinson both flocculation and nitrogen determination indicate that

practically no free toxoids exist within the equivalence zone, even when the proportion of antitoxin to toxin units is as 300 to 450. It seems quite definite, therefore, that the actual explanation of the L_0 to L_+ phenomenon is to be found in the "multiple proportion" phenomenon still further confirmed by Eagle (16), from which it is quite clear that, after a neutral point has been reached, considerable amounts of additional toxin may be fixed by a constant amount of antitoxin. The exact proportions of such multiple unions cannot of course be precisely determined. But from the ratios obtained by Pappenheimer and Robinson, taking TA as the ratio at the point of antitoxin excess, it would be T_2A at the flocculation point and approach T_3A in the neighborhood of the toxin excess end.

The Danysz Phenomenon. On the basis of our present knowledge of the manner in which toxin and antitoxin react, the Danysz phenomenon loses much of its former mystery. It will be remembered that Danysz noticed that when toxin is added to antitoxin in quantities so measured that a neutral mixture should result, neutrality is achieved only when the toxin is added to the antitoxin all at one time; whereas when the toxin is added in several fractions with considerable intervals between each addition, the resulting mixture is highly toxic. This observation was confirmed for diphtheria toxin by von Dungern (17) and for megatheriolysin and its antibody by Craw (18). Danysz quite correctly attributed the phenomenon to the ability of the toxin to unite with the antitoxin in multiple proportions. The issue was clouded for a time by the "toxoid avidity" controversies, but it would seem quite clear that recent investigations explain the phenomenon in terms of Danysz' original opinion. Thus it is plain, both from the investigations of Healey and Pinfield (14) and from those of Pappenheimer and Robinson (15) that when toxin is added to excess antitoxin, each toxin equivalent can react with and bind approximately 2 antitoxin equivalents. Thus, in the table given on page 191, the first 50 equivalents of toxin added to 300 equivalents of antitoxin remove something over 100 antitoxin equivalents, even though there was no visible flocculation. Healey and Pinfield's results are entirely consistent with this, so that for the "antitoxin excess" reactions they write their conception of the union in the form of "TA_2." It is self-evident that if the first fraction of toxin removes considerably more than its equivalent of antitoxin, there must be less free antitoxin left for neutralization of the subsequently added amount of toxin. The toxicity of the mixture is therefore inevitable.

The immediate toxicity of such mixtures is clear and demonstrable, but if the toxin-antitoxin union is a reversible one which comes to equilibrium for each particular mixture according to the relative amounts of toxin and antitoxin present, we should expect that, with time, the mixtures originally toxic would gradually become neutral. This is

actually the case. Healey and Pinfield have shown that the full theoretical toxicity of mixtures made by the Danysz technique can be found only if measurements are made almost immediately. Toxicity diminishes slowly and at the end of the fourth day has usually disappeared. It is still to be remembered, however, that reversibility of the reaction may not always be complete. When flocculation has occurred, the floccules may at times be somewhat insoluble. They are usually soluble in an excess of one or the other of the reagents, bu in most cases a considerable excess either of toxin or antitoxin must be added to flocculated mixtures before the floccules are completely redissolved. According to Pappenheimer and Robinson, it is a fact that toxin and antitoxin combine in multiple proportions, and the rearrangement of each successive intermediate complex to a more stable form explains the Danysz effect. Unless we assume this *second* reaction, multiple proportions will not explain the Danysz effect which occurs in regions of antitoxin excess when no flocculation or visible aggregation ever occurs.

It is not at all unlikely that in dealing with reactions in which materials of great molecular size are involved, mass action phenomena, while holding good in principle, may be modified to some extent by the relatively immense sizes of the united complexes which, being proteins, may become "denatured" — less soluble — and therefore only partly predictable by mass action formulae.

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CHAPTER VII

THE BACTERICIDAL PROPERTIES OF BLOOD SERUM. SENSITIZATION. ALEXIN

A POSSIBLE relationship between the blood and the defenses of the body against infection was foreshadowed by observations made as early as 1792 by John Hunter. In his "Treatise on the Blood, Inflammation and Gunshot Wounds," he noted that the blood did not decompose as readily as other putrescible material. A century later, Traube (1874) expressed the opinion that blood could destroy bacteria. Similar observations were made by Lister and by Grohmann (1) but no experimental work was carried on until 1886, when the subject was taken up by Nuttall (2), von Fodor (3), Flügge and Buchner (4). These investigators working with defibrinated blood, peptone blood, and blood serum, showed that such substances all exerted measurable destructive influence upon bacteria, and Nuttall found that this bactericidal power was weakened on standing, and could be destroyed by heating to 60° C.

Their method consisted in planting controlled amounts of various bacteria in measured quantities of blood and, after several hours at 37° C., pouring plates, thus determining the numbers of surviving organisms. There was at first much difference of opinion as to the mechanism responsible for the destruction of the bacteria.

Early theories were formulated chiefly upon the underlying thought that the animal body was primarily passive in its relation to the invading microorganisms, and that the disappearance of bacteria from the body was due to chemically or physically unfavorable environment which prevented multiplication. Thus Billroth (5) believed that bacteria could thrive in the body only after preceding putrefactive change. Others attempted to discover a relation between the degree of alkalinity of the blood serum and the destruction of bacteria. (See also Baumgarten's "Osmotic Theory" (5 a).) These "Passive" theories of immunity, however, lost value as soon as studies on the mechanism of bacterial destruction began to make headway. The part played by the cells had been emphasized by Metchnikoff. The humoral theory was conceived by Buchner, as the first important theoretical result of Nuttall's discovery. Buchner looked upon the antibacterial power as depending upon a constituent of the fresh blood plasma, which he named "*alexin*"

(protective substance), and which he believed to be comparable to a proteolytic enzyme. The analogy to ferment action was formulated because of the heat sensitiveness and instability of the bactericidal substance; he suggested that this alexin might possibly be a product of the tissue or blood cells, possibly leucocytic in origin.

Buchner found that the action of the alexin upon bacteria was most marked at the temperature of the body, and that it was capable of destroying bacteria in the subcutaneous tissues and the serous cavities of the animal body, without the aid or co-operation of cellular elements.

The Pfeiffer Phenomenon. Pfeiffer (6, 6a, 6b, 6c), studying the bactericidal effect in cholera immunity, found that the injection of cholera spirilla into the peritoneal cavity of a guinea pig which had recovered from a cholera infection was followed by a rapid destruction of the bacteria. If small quantities of exudate were taken out of the peritoneum at varying intervals after injection, granular change and swelling of the spirilla were followed, soon after, by complete dissolution and disappearance. Such animals would recover from doses of bacteria which, in control animals of the same weight, caused death. The phenomenon was specific, in that the dissolution of organisms occurred only in the cholera-immune animals. In other words, the guinea pig had acquired a specific antibacterial power, expressed by the process of "bacteriolysis," a property possessed to only a very slight extent by the peritoneal exudate of normal animals. It was the next step to determine whether the bacteriolytic power could be transferred to the peritoneal cavity of a normal animal by injecting, together with the bacteria, a small amount of the serum of an immune animal. This was found to be the case and, although such immune serum, like normal serum, was deprived of its *in vitro* bactericidal power by heating, Pfeiffer found that heated serum is quite as active as fresh immune serum when the experiment is done *in vivo* instead of *in vitro*. We may summarize these experiments as follows:

1. Dissolution of cholera spirilla takes place in the peritoneal cavity of a cholera-immune guinea pig. Similar lysis takes place not at all, or only to a slight extent, in the peritoneum of a normal pig.
2. The protection is specific.
3. The protection may be transferred from an immune to a normal guinea pig, by injecting immune serum together with the bacteria. In a normal animal so treated lysis is in every way similar to that observed in the immune.
4. The transfer of the lytic power can be brought about by heated serum.

Of the phases of this "Pfeiffer phenomenon" the one most difficult to understand was the transference of the lytic property with the heated

serum. Pfeiffer took this to signify that the destruction of some bacteria could take place entirely without the phagocytic participation of the body cells, a view in sharp contrast to that of Metchnikoff. He assumed, however, that the co-operation of the living tissues was necessary, a possible activation by substances derived from the endothelial cells lining the peritoneal cavity.

A protocol of the Pfeiffer phenomenon follows:

The quantity of bacteria used must be chosen with some regard to the virulence and toxicity of the particular culture employed, since protection of animals by bactericidal or bacteriolytic sera does not follow the law of multiple proportions as in the case of the protection against toxins by antitoxins. While the dose of bacteria chosen should be considerably above the minimal lethal dose for an animal of the weight used, it should be remembered that the bactericidal serum does not possess antitoxic properties against the poisons liberated, the bacteria undergo dissolution, and protection by bacteriolysis is limited to a definite maximum of bacteria, beyond which no increase of serum will avail. The following table illustrates an experiment of this kind.

PFEIFFER PHENOMENON

WEIGHT OF GUINEA PIG	DOSE OF BACTERIA * CHOLERA SPIRILLA	AMOUNT OF INACTIVATED IMMUNE SERUM	RESULT
(1) 215 gm.	2 mg.	0.1 cc. in 1 cc. salt solution.	Complete dissolution in less than 1 hour. Lives.
(2) 230 gm.	2 mg.	0.05 cc.	About the same as first.
(3) 200 gm.	2 mg.	0.01 cc.	Somewhat slower than in other two; a few unchanged spirilla after 1 hr. Final dissolution. Pig lives.
(4) 245 gm.	2 mg.	0.005 cc.	Similar to (3) but complete dissolution in 2 hr. Pig lives.
(5) 220 gm.	2 mg.	0.001 cc.	After 30 min. the spirilla seem to have begun to multiply. Dies with innumerable active spirilla in peritoneum.
Normal control			
(6) 210 gm.	2 mg.	0.1 cc. normal inactive rabbit serum.	Very slight lysis at the beginning. Soon rapid multiplication. Dies.

* The bacteria may be measured for such an experiment by standard loopfuls (1 loop being equal to 2 milligrams), or by volume in emulsion with salt solution.

The cardinal points of Pfeiffer's phenomenon were confirmed, but his assumption that the process could take place only within the animal body was disproved by both Metchnikoff (7) and Bordet (8). These

investigators succeeded in obtaining extracellular lysis of cholera spirilla in hanging-drop preparations. The former produced the phenomenon by adding to the hanging-drop preparations small quantities of extracts of leucocytes. The latter disproved the necessity of cellular intervention and explained the activity of the heated immune serum within the peritoneum as follows:

Reactivation. As we have seen, the bacteriolytic properties of immune serum are destroyed by heating to from 50° to 60° C. In studying this phenomenon Bordet discovered that the addition of a small amount of fresh normal serum completely restored the original bacteriolytic power to the heated immune serum. There are thus two distinct serum elements necessary. Fresh normal serum by itself has very slight or no bacteriolytic power. Fresh immune serum has powerful and rapid effects. Heated immune serum loses this power completely, but this is restored by the addition of fresh normal serum. Furthermore, the specific nature of the bacteriolysis by the immune serum is unchanged after it has been inactivated by heat and subsequently reactivated. The inference is plain. Immunization of an animal incites the production, in the blood, of a "preventive" substance, which is moderately resistant to heat, and which is specific for the bacteria employed in the immunization. This substance by itself does not destroy the bacteria, but depends for its effectiveness upon the co-operation of another substance present universally in normal serum, which is non-specific, corresponds to Buchner's alexin, and is apparently not increased by the process of immunization.

Hemolysis. The process is most easily made clear in connection with hemolysis. Bordet (9) prepared immune serum in a goat by injection of cholera spirilla. As normal serum he had used guinea-pig serum, and the latter frequently contained a few blood corpuscles. He noticed that these corpuscles were frequently clumped in the goat serum and correlated this with bacterial agglutination. It occurred to him, therefore, that the phenomena both of agglutination and of lysis might be expressions of general biological laws, not limited to bacteria. Accordingly he injected rabbit blood into guinea pigs, and examined the serum of animals so treated for its action upon rabbit corpuscles, *in vitro*, and found that the sera of "blood-immune" animals acquired not only agglutinative power against the corpuscles injected, but also specific "hemolytic" powers, that is, the property of causing a solution of hemoglobin out of the red cells. The hemolytic power was lost if the serum was heated to from 50° to 60° C., but could be completely restored by the addition of a little fresh normal serum which in itself possessed no hemolytic properties for the given species of cell. The specificity of the phenomenon resided entirely in the heat-stable factor. The heat-

sensitive or "alexin" factor was non-specific, and not increased during the process of immunization.

Similar observations were made independently, in the same year, by Belfanti and Carbone, and confirmation was obtained in the year following by von Dungern, and by Landsteiner.

After Bordet had thus established the important fact that hemolysis was in every way analogous to bacteriolysis, Ehrlich and Morgenroth (10, 10 a, 10 b) undertook the study of the mechanism of hemolytic phenomena, hoping thereby to elucidate the mechanism of lysis in general. Hemolysis obviously lends itself more easily to experimentation than does bacteriolysis.

Ehrlich and Morgenroth accordingly inactivated (at 56° C.) a goat serum which was hemolytic for beef blood, left it in contact with beef blood corpuscles for 15 minutes at 40° C., and then separated the cells from the supernatant fluid by centrifugation. To the blood cells they then added a little normal goat serum (by itself not hemolytic for beef blood) and found that complete hemolysis occurred.

They then investigated the mechanism of the reaction by experiments like that following:

EXPERIMENT TO SHOW THAT THE ANTIGEN (IN THIS CASE RED BLOOD CELLS) ABSORBS THE SPECIFIC HEAT-STABLE ANTIBODY OUT OF THE IMMUNE SERUM

In a test tube { 4 cc. of 5 per cent emulsion of washed beef blood.
1 cc. of inactivated blood serum of a goat treated with beef blood.

These substances are left together at 37.5° C. for one hour and then centrifugalized into:

I	II
Sediment of Corpuscles. — To this are added 4 cc. salt solution and 0.8 cc. fresh normal goat serum, by itself not hemolytic for beef corpuscles. Result = *Complete hemolysis.*	*Supernatant Fluid Containing the Serum and Salt Solution.* — To this are added washed beef corpuscles and 0.8 cc. fresh normal goat serum. Result = *No hemolysis.*

This experiment has the following significance: the fresh serum of the goat, previously injected ("immunized") with the beef blood, possessed the property of dissolving the hemoglobin out of beef corpuscles, viz., hemolyzing them. Heating this serum to 56° C. for 20 minutes deprived the serum of all hemolytic power, *i.e.*, inactivated it. The addition of a little fresh goat serum, in itself inactive, completely reactivated the hemolytic properties of the heated immune serum. This shows that

hemolysis is a dual process in which a heat-sensitive and a heat-stable substance co-operate, neither of them capable of producing lysis by itself. The heat-sensitive ingredient, corresponding to Buchner's "alexin," is present in normal serum. The heat-stable substance, specific and increased in immunization, is fixed to the red corpuscles, and renders them susceptible to the action of the heat-sensitive substance in the normal serum.

In attaching to this heat-stable element, the blood cells remove it from the solution.

It was also shown that *only* the heat-stable substances could be fixed by the red cells, while the heat-sensitive "alexin," which Ehrlich now called "*complement*," could not attach to the unsensitized red cells. For if such complement, in the form of fresh serum, was added to washed red blood cells, and the mixture after standing at 40° C. for some time was centrifugalized, the complement remained in the supernatant fluid.

EXPERIMENT TO SHOW THAT COMPLEMENT OR ALEXIN IS NOT ABSORBED BY UNSENSITIZED CELLS

Mixed in a test tube { 4 cc. of 5 per cent emulsion of washed beef blood. 0.8 cc. of fresh normal goat serum (alexin or complement), not, by itself, hemolytic for beef blood.

These substances are left together at 37.5° C. for one hour, then centrifugalized into:

I	II
Sediment of Cells. — To this is added *inactivated serum* of immune goat which would cause hemolysis if alexin were present.	*Supernatant Fluid* (salt solution and serum). — To this is added washed beef blood and inactivated serum of immune goat containing heat-stable element.
Result = *No hemolysis.*	Result = *Complete hemolysis.*

The fact that the alexin or complement is used up during processes of lysis has been made the subject of repeated investigation. Muir (11) comes to the conclusion that the complement is used up in hemolysis, but that it does not always disappear completely, this depending upon the relative amount of sensitizer present. Liefmann and Cohn (12) believe that the disappearance of free complement from hemolytic complexes is due to:

(1) fixation by the products of hemolysis (stromata, etc.) after the reaction is accomplished,

(2) dilution, and

(3) to weakening because of prolonged exposure to dilute solution at 37° C.*

Briefly reviewed, the mechanism of hemolysis and bacteriolysis is as follows: The antigen (blood cells, bacterial cells, etc.) undergoes hemolysis or bacteriolysis when acted upon by two factors, one a thermostable substance, specific and increased during immunization, the other a thermosensitive substance present in fresh serum, not increased (13) by immunization of the animal and not specific. The thermostable substance becomes specifically united with the antigen regardless of the presence or absence of the alexin or complement. The union takes place even at 0° C. The alexin or complement, however, cannot enter into relation with the antigen unless this has been rendered susceptible to it by attachment to the thermostable specific substance. When this has taken place, union with complement occurs, but only at temperatures above 0° C. (the speed and completeness of the union increasing as the temperature approaches 40° C.). The result of the union is lysis or, in the case of bacteria not easily soluble, the bactericidal effect.

The "Amboceptor" Conception. Ehrlich conceived sensitization as a mediation on the part of the heat-stable substance between the antigen and the alexin or complement. He called the sensitizer "amboceptor" (twin-receptor), because of its mode of action, and was conceived as possessing two combining groups — one the "cytophile," by means of which it is anchored to the sensitive cell, the other the "complementophile," by means of which it exerts affinity for the complement. The original cell receptor, from which such an "amboceptor" takes its origin, is one which not only can combine with the antigenic substance offered for assimilation, but which also possesses another atom group by means of which it can establish union with the alexin or complement. In spite of much ingenious experimentation, however, the "amboceptor" nature of sensitizer has never been demonstrated; and millions of "complement-fixation" tests have failed to reveal any evidence of the postulated "complementophile" group. The assumption is, moreover, no longer necessary to explain observed phenomena in view of the studies on antigen-antibody union outlined in another chapter.

Multiplicity or Singleness of Alexin. A question of fundamental importance is that regarding the unity or multiplicity of alexin or complement. Here again there has been some misconception as to the meaning of those who maintain the unity of alexin. Neither Bordet, nor anyone else familiar with experimental conditions, has ever maintained that the alexins of different animals were functionally identical. It is a well-known fact that the fresh blood sera of various animal species differ

* In the ordinary dilution used in Wassermann tests, the unit of complement employed may deteriorate entirely within several hours at 40° C.

from each other considerably in their power to activate bactericidal or hemolytic systems. Fresh guinea pig serum, for instance, is powerful in activating many sensitized blood-cell complexes, but weak in activating sensitized guinea pig corpuscles. Often one finds that the alexin of an animal is but weakly capable of producing hemolysis of the sensitized cells of its own species, though this is not a general rule.

Again, even without such species relationship, a given alexin may be very weak for certain complexes and strong for others. The alexin of horse blood can even be fixed to sensitized cells without producing much, if any, hemolysis (14). An alexin which may be strong for a given hemolytic complex may be weak for certain bactericidal complexes, or *vice versa.*

In a study of the antiserum-complement bacteriolysis of "smooth," virulent, meningitic strains of *H. influenzae,* Dingle, Fothergill, and Chandler (14 a) found that complement of different species of animals did not activate antibody produced by active immunization in other species. Guinea pig complement failed to activate rabbit and horse antiserum. Rabbit complement activated guinea pig immune serum but not horse immune serum. Curiously enough, human complement (infants' serum being used because of the presence of natural *H. influenzae* antibody in the serum of older individuals), activated guinea pig, horse and rabbit immune sera. These observations are of considerable importance for the treatment of patients with antibacterial sera, as in *H. influenzae* meningitis.

During the course of these experiments it was observed that guinea pig immune serum, although practically devoid of demonstrable agglutinins and precipitins, exerted a surprisingly effective bactericidal action *in vivo.* Continued immunization of these animals has produced a slow increase in bactericidal titer without the development of appreciable agglutinins or precipitins. Thus, the reputation of these animals as being unsuitable for antibody production may be dependent not on a lack of antibody formation, but on the inability of the antibodies to produce visible agglutination or precipitation.

These experiments can best be discussed together with a review of the literature reporting the studies of other investigators, particularly in regard to the variation in activity of different complements with a given antibody in bactericidal systems, similar differences in hemolytic systems, and the question of the identity of the complement taking part in both reactions.

The following review of this problem is taken directly from the discussion by Dingle, Fothergill, and Chandler.

Since Bordet (15) demonstrated the *in vitro* lysis of the cholera vibrio by heated immune serum which was reactivated by fresh normal serum,

a long series of investigations on complement action have been reported. Wassermann (16) noted that, although fresh guinea pig serum did not restore the hemolytic activity of heated normal goat serum for guinea pig red blood cells, it did restore the bactericidal activity of the heated serum of the same goat after immunization with cholera vibrio.

Wechsberg (17) found that a mixture of active normal guinea pig serum and inactivated normal rabbit serum was capable of killing the typhoid bacillus, which neither of the sera alone would do. He further showed that normal active rabbit serum restored the bactericidal action of inactivated immune rabbit serum for *Vibrio Metschnikovii*, whereas normal active pigeon serum was without effect. Injection of this inactivated immune rabbit serum into pigeons did not protect them against a fatal dose of the vibrio. When, however, normal active rabbit serum was injected with the inactivated immune serum, the pigeons survived. It is interesting to note that a prozone was obtained in the latter part of this experiment.

The pigeons receiving 1.0 cc., 0.3 cc., 0.1 cc., and 0.01 cc. of immune serum with 1.0 cc. of normal active rabbit serum died, whereas those receiving 0.03 cc., 0.01 cc., and 0.003 cc. of immune serum and 1.0 cc. of normal rabbit serum survived. It is also interesting to note that 1.0 cc. appeared to complement the antibody *in vivo* even though considerable dilution must have occurred when this amount was injected into a pigeon. Pigeons were then immunized with killed cultures of *V. Metschnikovii*. Both the fresh immune pigeon serum and the heated serum reactivated with normal pigeon serum exerted some bactericidal action as indicated by colony counts of the mixtures, although viable organisms were present in all of the serum dilutions. The immunized pigeons, however, remained alive and well after the injection of many times the fatal dose of living organisms. These results were explained by postulating that the amboceptor of pigeon immune serum was different in its complementophilic group from the amboceptor of rabbit immune serum, and that a suitable complement was necessary for each amboceptor.

Thjötta (18), in a study of the Neisser-Wechsberg phenomenon in bactericidal immune serum, used the sera of rabbits immunized with dysentery bacilli and both guinea pig and human serum for complement. No mention is made of any difference in the ability of either serum to complement the immune rabbit serum. In Pandit's (19) experiment comparing the bactericidal action on the cholera vibrio of immune rabbit serum with rabbit, guinea pig, and human complements, no significant difference can be seen.

Mackie and Finkelstein (20) studied the mechanism of natural bactericidal action by the normal serum of various animals (ox, sheep, horse,

rabbit, guinea pig, rat, and man) on *B. typhosus*, *B. dysenteriae* Shiga, *B. proteus*, and *V. cholerae*, and stated that the complements of different animal species were found to be interchangeable in these bactericidal reactions. On the other hand, Shrigley and Irwin (21) found that guinea pig serum was not able to activate the normal thermostable bactericidal antibody of ox, rabbit, or sheep serum against *Br. suis*, although it was active in hemolysis against chicken red blood cells and anti-chicken red cell rabbit serum. Rabbit complement activated only heated rabbit serum.

Ward and Wright (22), as previously stated, using anti-*H. influenzae* horse serum, obtained bactericidal action with fresh normal rabbit blood, fresh human serum, and fresh guinea pig serum. The fresh guinea pig serum was more effective than fresh human serum ; in fact, in a hemolytic system, the guinea pig serum was four times more active than the human serum. Wilkes-Weiss (23) obtained killing of *H. influenzae* using guinea pig serum as complement with both unconcentrated and concentrated horse antiserum.

It is difficult to reconcile these results with those obtained by Dingle, Fothergill, and Chandler. Technical differences may account in part for the results obtained by Wilkes-Weiss since she used 24-hour cultures, and incubated the serum-organism mixtures only 3 hours, which may allow errors due to agglutination as pointed out by Gengou (24). The sera used were not particularly potent, moreover, since the maximum number of organisms killed in any dilution of concentrated serum was only 18,500 and, in the unconcentrated serum, 1850. The only apparent technical differences between Ward and Wright's experiments and those of Dingle, Fothergill, and Chandler are the age and virulence of the cultures.

That complements from different species vary considerably in their reactions with different antibodies has been frequently demonstrated in hemolytic systems. Bordet (13) observed that inactivated anti-rabbit red cell guinea pig serum was completely activated by fresh normal guinea pig serum but incompletely by fresh rabbit blood, which he believed was due to the great number of red blood cells in such blood. Injection of the immune guinea pig serum intravenously into rabbits produced fatal results, however.

Ehrlich (25) pointed out that normal guinea pig serum would restore the hemolytic activity of heated dog serum for guinea pig red blood cells. In a later publication with Morgenroth (26), he compared the quantities of anti-ox red cell rabbit and goose sera required to give lysis with various complements. Guinea pig, rabbit, rat, goose, and hen complements produced lysis with both immune sera. Goat complement was lytic with rabbit but not goose immune serum, whereas pigeon complement

was active only with the goose serum. Fresh horse complement activated neither of the sera, nor was it effective with goat amboceptor. It was not devoid of complement, however, since 0.15 cc. lysed guinea pig red blood cells almost completely, although it was inactive for rabbit red blood cells. In order to explain these results, Ehrlich postulated a multiplicity of complements which are capable of reacting only with suitable amboceptors.

Marks (27) demonstrated that heterologous guinea pig and hog complements both activated anti-sheep red cell and anti-ox red cell amboceptors, whereas the homologous sheep and ox complements were ineffective.

In an antihuman red blood cell hemolytic system, Noguchi and Bronfenbrenner (28) found that guinea pig complement gave lower titers with rabbit and guinea pig amboceptors than did rabbit complement, whereas the reverse was true with dog and cat amboceptors. Chicken amboceptor had no action with either guinea pig or rabbit complements.

Muir (29) studied the activation with various complements of antisera prepared with ox red corpuscles in the rabbit, cat, goat, duck, and ox (isolysin). With all the antisera, except that of the duck, hemolysis was obtained with all complements except that of the ox, pigeon, and horse. The latter complement, however, was effective with cat antiserum. Muir points out that hemolysis depends chiefly on the complement and the corpuscles used. The failure of sensitized corpuscles to lyse with a given complement may be due to non-combination of the complement or to insensitivity of the corpuscles to the action of that complement even though it may be fixed. "For example, ox's corpuscles sensitized by the immune body of the rabbit are not lysed by horse's complement, yet it is found by the deviation or subtraction method that a considerable amount of the latter has entered into combination; in other words, complementing without lysis has occurred."

Mackie (30) observed that the globulin fractions of both rabbit and human sera displayed practically the full complementing action of the serum with anti-ox red cell rabbit serum in contradistinction to guinea pig complement which required both the albumin and globulin fractions. Fresh horse serum did not produce lysis. Furthermore, the globulin fractions of rabbit and human sera produced hemolysis with venomized (cobra) ox red cells, although the whole sera were inactive due to the inhibiting action of the albumin fractions. Horse globulin and albumin were both active, the action of the former being due to a heat-labile complement body, that of the latter to lecithin.

In a study of the serum from a complement-deficient strain of guinea pigs, Hyde (31) reported that activation of the deficient serum occurred with fresh human and guinea pig sera, to a less extent with dog, rabbit,

and cat sera, and not at all with the sera from a large number of other species. After heat inactivation, only the human serum retained its full activating power, five times as much guinea pig serum was necessary for activation, and the other sera were without effect.

Capart (32), in studying the hemolysis of sensitized red blood cells with homologous and heterologous complements, found that only human complement would exert the same action against its homologous as against heterologous cells, whereas 64 times as much guinea pig complement as rabbit complement was required to lyse sensitized guinea pig red blood cells. He further evaluated numerically the activity of the complements as follows: guinea pig, 32; human and rabbit, 4; and sheep, 1; *i.e.*, 32 times as much sheep complement was required to give the same result as guinea pig complement.

Da Costa, Cruz, and Penna (33) reported that guinea pig complement was most active in lysing sheep red blood cells sensitized with 30–40 units of rabbit hemolysin, rabbit complement was the least active, and human, monkey, and dog complements were intermediate.

These results demonstrate the variations in the hemolytic action of complements from different species. Which of the numerous explanations is correct does not immediately concern us here; the important fact is that this situation does exist with hemolytic complement and, as has been shown by others as well as by Dingle, Fothergill, and Chandler, with bactericidal complement.

This brings up the question of the identity of the complement taking part in these two reactions. From a review of the literature it is impossible to determine whether or not the two are identical or whether there is but a superficial resemblance between them. With a given complement, manipulations destroying or removing mid-piece, end-piece, third component (34), or fourth component (35), and possibly Congo red inhibition (36), destroy both bactericidal and hemolytic complements, and both are restored to an equal degree by the same procedures. The differences noted in the two (36, 37, 38, 39) are probably of a quantitative nature. On the other hand, there is a definite difference in the behavior of a complement in bactericidal and hemolytic systems, so much so in the case of *Br. suis* (21) that the complements arranged according to activity were in the reverse order. It is not necessary to postulate qualitative differences to account for these results, however, since they may be due to differences in the quantitative relations of the various complement components or to the resistance of the cells or bacteria to lysis or destruction, as Muir (29) has pointed out with sensitized ox red cells and horse complement — the complement was fixed but lysis did not occur. In the case of the influenza bacillus, Pittman and Goodner (40) have obtained complement fixation with rabbit antisera and guinea

pig complement. We have obtained similar results with rabbit antiserum and guinea pig complement which were incapable of killing the bacillus in bactericidal experiments.

The variation in the activation of antibodies by complement of different species is of considerable practical importance in diagnostic tests and in therapy. Ehrlich (25) pointed out that ". . . in therapeutic application of antibacterial sera to man, therapeutic success is only to be attained if we use either a bacteriolysin with a 'complement' which is stable in man, or at least a bacteriolysin, the 'immune body' of which finds in human serum an appropriate 'complement.'" This point has been reiterated by Wassermann (16) and Wechsberg (17).

Ehrlich and Morgenroth (41) and Ehrlich and Sachs (42) brought forward evidence from which they deduced the existence of a number of different alexins or complements, for hemolytic complexes, in the same serum. The earlier experiments of Ehrlich and Morgenroth on this question were carried out by means of the filtration of normal goat serum through Pukall filters (43, 43 a); it appeared that the serum which passed through the filters was complementary for sensitized guinea pig cells, while that part which had, in the original serum, hemolyzed sensitized rabbit cells was left behind. Similar differentiation of complement they later based upon experiments with anticomplementary sera which, they showed, did not equally neutralize all the complementary functions of a serum. In support of their contention Neisser (44) described two complementary substances in rabbit serum, the one active for bactericidal complexes, the other for hemolytic, and similar experimental evidence has been brought forward by Wassermann (16) for guinea pig and by Wechsberg (17) for goat serum. The evidence advanced is based chiefly on experiments in which it was found that a normal serum which possessed both bactericidal and hemolytic powers could be deprived of the complement for one or the other of these activities only, by absorption with the respective cells. In addition to this, Ehrlich and Morgenroth, Ehrlich and Sachs (45, 45 a), Wendelstadt (46), and others, claimed to have differentiated various complements in the same serum by heating, by the action of weak acids or alkalies, or digestion of sera by papain.

Wilde (47, 48) refuted the experiments of Neisser, who claimed that the absorption of fresh rabbit serum with anthrax bacilli deprived this serum only of its bactericidal but not of its hemolytic complement (49). Wilde showed that, if a sufficient excess of anthrax bacilli (or of typhoid bacilli or cholera spirilla) were added, both bactericidal and hemolytic complement could be absorbed from normal serum. He concludes that there is actually only one alexin present, but that the red cells and anthrax bacilli differ in their susceptibility to this alexin (or, in other words, that the sensitization of these cells by the normal serum is unequal), a conclusion which seems

rational in view of the fact that one and the same complement may differ greatly in the degree of its activity upon different sensitized complexes.

Bordet found that absorption of normal serum with unsensitized cells rarely deprived this serum of all of its alexin, even when these cells were used in considerable amounts. This he attributed to the feeble sensitization of the cells. If, however, strongly sensitized cells were added to such a normal serum, all the alexin would be taken up. He refers the phenomenon of specific alexin absorption, observed by previous workers, to insufficiency in the sensitization of the cells used in the preliminary exposure.

Zone Phenomena in Bactericidal Tests.* The so-called "Zone Phenomenon" which will be discussed in greater detail in connection with the precipitin reaction was first clearly described in observations on bactericidal tests made by Neisser and Wechsberg. An experiment illustrating the "Neisser-Wechsberg" phenomenon is as follows:

In carrying out such measurements, it is customary to add in series, to constant amounts of bacteria, varying amounts of inactivated antiserum and constant amounts of complement or alexin. These mixtures are set away in the thermostat for 3 to 4 hours, are then mixed with agar and plates are poured. The colonies will give an indication of the number of bacteria killed in each mixture when compared with similar plates poured from tubes in which the same amounts of bacteria had been mixed with alexin alone. The following table will exemplify such a test:

Typhoid Bacilli	Typhoid Antiserum Inactive	Alexin	Result in Colonies After 3 Hours' Incubation
Constant quantity	.1 cc.	.07 cc.	Many thousand
Constant quantity	.01 cc.	.07 cc.	Many thousand
Constant quantity	.005 cc.	.07 cc.	150 colonies
Constant quantity	.001 cc.	.07 cc.	200 colonies
Constant quantity	.0005 cc.	.07 cc.	800 colonies
Control I, constant quantity . .	—	.07 cc.	Many thousand
Control II, constant quantity . .	—	—	Many thousand

Although there has been considerable bactericidal action in the mixtures in which 0.005, 0.001, and 0.0005 cc. of antiserum were used, in the mixtures in which as much as 0.1 and 0.01 cc. were present, and in which one would naturally expect a still greater antibacterial action, the contrary occurred.

Neisser and Wechsberg believed that their observation confirmed Ehrlich's supposition of a complementophile group of the amboceptor, since they assumed that the failure of bactericidal effect in the more concentrated serum was due to an absorption of complement by the excess amboceptor which had not united with bacteria and was therefore ineffective. This they called "complement deviation." Their assumption, however, is shown to be entirely erroneous, since similar zone

* See discussion of Zone Phenomenon in chapter on Antigen-Antibody Union.

phenomena are observed, in agglutinin and precipitin reactions in which no alexin participates. A practically important observation on the zone phenomenon in bactericidal tests with whole blood, where phagocytosis doubtless plays a role, is the following one, obtained by Ward with defibrinated human blood, pneumococci, and horse antiserum.

INOCULATION OF 30,000 DIPLOCOCCI *

CONCENTRATION OF SPECIFIC SOLUBLE SUBST.	CONCENTRATION OF ANTISERUM	RESULT
1/75,000	1/20	+ +
1/75,000	1/80	+ +
1/75,000	1/320	+ + + +
1/75,000	1/1280	+ + + +
1/75,000	1/5000	0
1/75,000	1/20,000	0
1/75,000	1/80,000	0
1/75,000	1/320,000	+ + + +
1/75,000	1/1,128,000	+ + + +
1/75,000	——	+ + + +
——	——	

++ = growth of pnuemococci.

Quantitative Relationship of Sensitizer and Alexin. Morgenroth and Sachs (51) were the first to study systematically mutual quantitative relations between complement and amboceptor. They showed that the more highly cells were sensitized, the smaller was the quantity of complement which was needed for their hemolysis, and vice versa. The following extract from one of their protocols will illustrate this:

BEEF BLOOD CELLS 5%, 1 CC., ANTIBEEF GOAT SERUM, GUINEA PIG COMPLEMENT

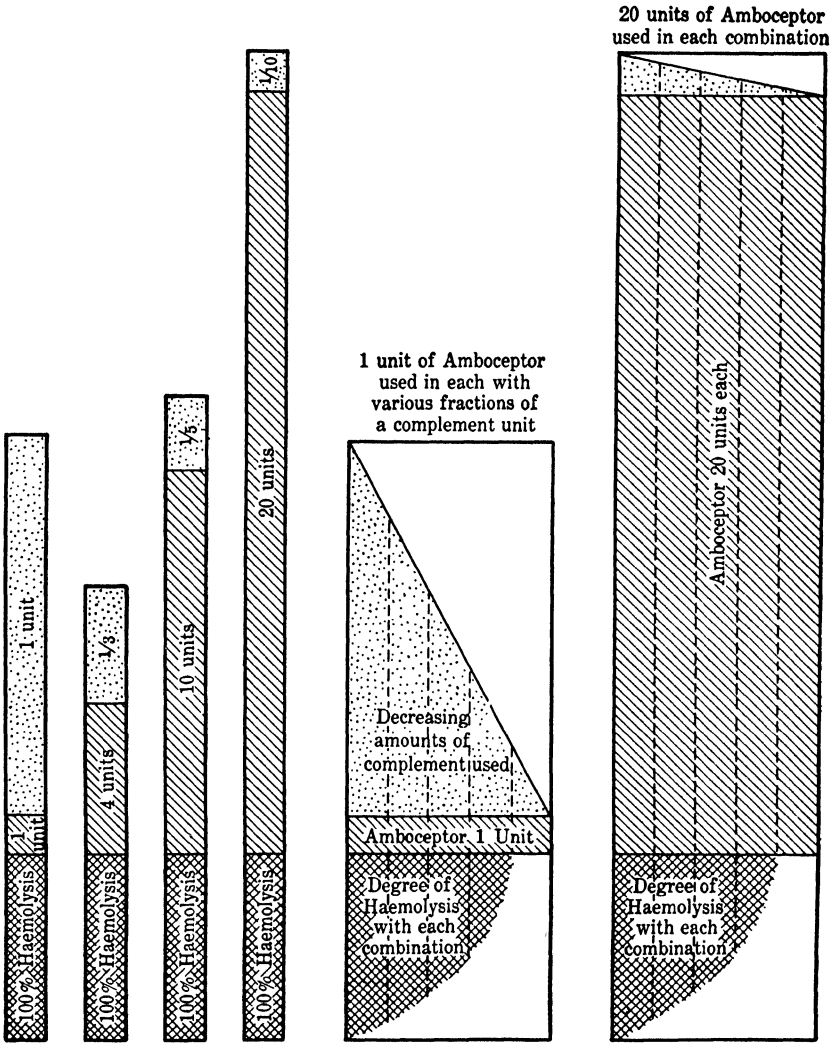
AMOUNT OF AMBOCEPTOR	RELATIVE AMOUNT OF AMBOCEPTOR	AMOUNT OF COMPLEMENT FOR COMPLETE HEMOLYSIS
.05	1	.008
.2	4	.0025
.4	8	.0014

This bears out Bordet's views, since, knowing the differences in functional efficiency of various complements for different hemolytic and bactericidal complexes, we could expect that insufficient sensitization of an antigen might fail to absorb alexin completely out of the serum, thus giving a negative result which would simulate complete lack of affinity.

* Taken from Ward (50).

The quantitative relations here outlined have been diagrammatically represented by Noguchi.

The Conglutinin Effect. An observation reported by Ehrlich and Sachs (42) seemed to represent, as they express it, an "Experimentum Crucis" proving Ehrlich's contention of the intermediary function of the amboceptor in contrast to Bordet's "sensitization" idea. The facts, as they record them, are as follows: When fresh horse serum is added to guinea pig cor-



NOGUCHI'S DIAGRAM ILLUSTRATING THE QUANTITATIVE RELATIONS BETWEEN ANTIGEN, AMBOCEPTOR, AND COMPLEMENT

(Taken from Noguchi, "Serum Diagnosis of Syphilis," Lippincott, Philadelphia, 1910.)

puscles, slight hemolysis results. When inactivated ox serum alone is added to such corpuscles, no hemolysis results. If the corpuscles are, on the other hand, exposed to the action of the inactive ox serum, together with fresh horse serum, hemolysis is brought about. Apparently the ox serum sensitizes (or furnishes amboceptor to) the guinea pig corpuscles, rendering them amenable to the action of the complement in the fresh horse serum. In other words, inactivated ox serum can be reactivated by the addition of fresh horse serum. From this one would expect that if the guinea pig cells were exposed to inactive ox serum, then separated from the serum by centrifugalization and fresh horse serum subsequently added, hemolysis would ensue. However, this was not the case. When the cells were so treated, it was found that they had not been sensitized, and, what is more, it could be shown that the ox serum so employed had lost none of its ability to produce strong hemolysis when added to another complex of cells and fresh horse serum. Ehrlich and Sachs argued that this experiment showed the ability of the amboceptor in the ox serum to unite with alexin independently. The relation to the cell occurred only after the union of the amboceptor in the ox serum and the complement in the horse serum had been established.

Bordet and Gay (52) studied the Ehrlich-Sachs phenomenon and confirmed the experimental data, but cast doubt upon their conclusions. They made an observation which had escaped the attention of the former investigators. Heated bovine serum has but slight agglutinating power for guinea pig corpuscles. Fresh horse serum agglutinates them slightly and slowly. On the other hand, a mixture of the two sera agglutinates them rapidly and completely. The bovine serum possessed an accelerating or fortifying influence upon the weak normal hemolysins and agglutinins in the horse serum. Bordet and Gay suspected that this property might be due to an undescribed substance, peculiar to bovine serum.

They experimented with guinea pig corpuscles, anti-guinea pig sensitizer (from a rabbit immunized with guinea pig blood cells), and guinea pig alexin. They found that sensitized guinea pig cells are hemolyzed by guinea pig alexin slowly and imperfectly, as is often the case when the alexin comes from the same animal species as the cells. When heated bovine serum was added to the complex of sensitized cells and alexin, rapid agglutination and hemolysis resulted. Their experiments may be tabulated as follows:

1. Cells + guinea pig alexin + heated bovine serum = no agglutination; very slight hemolysis on next day.
2. Cells + sensitizer + heated bovine serum = slight agglutination; no hemolysis.
3. Cells + sensitizer + alexin + bovine serum = powerful agglutination and complete hemolysis in 10 minutes.
4. Cells + sensitizer + alexin = very slight agglutination and incomplete hemolysis in 30 minutes.
5. Cells + sensitizer = slight agglutination; no hemolysis.

In tube (1) the slight hemolysis was due to the small amount of normal sensitizer present in the bovine serum, and the slight agglutination in tube (5) is referable to the agglutinating power of the sensitizer. In tube (3) powerfully accelerating effects are exerted both upon agglutination and hemolysis when bovine serum acts upon sensitized corpuscles in the presence of alexin.

Bordet and Gay's interpretation of the Ehrlich-Sachs phenomenon is as follows: "When guinea pig corpuscles are added to a mixture of the two sera they are affected by the sensitizer of the horse serum and, to a certain extent, by the sensitizer in the heated bovine serum. This second sensitizer is, however, superfluous. Its presence is not necessary. When sensitization is effected, the corpuscles fix the horse alexin. This alexin, however, has only slight hemolytic power. But once the corpuscles have become sensitized and laden with alexin they become able to attract a colloidal substance of bovine serum, which unites with them. This new substance produces two results: it causes the blood corpuscles to be more easily destroyed by alexin and also agglutinates them. Consequently clumping, followed by hemolysis, is observed."

Bordet and Gay assume that the action of the bovine serum is due to a new substance which they speak of as "bovine colloid." This substance resists heating to 56° C., is probably a protein, and has the property of uniting with cells that are laden with sensitizer and alexin, but remains free in the presence of normal or merely sensitized cells.

This "colloid" is removed from bovine serum by absorption with sensitized bovine corpuscles which have been treated with horse alexin.*

Bordet and Streng (53) later studied this "colloid" more thoroughly and have suggested for it the name "*conglutinin.*" Streng (54) showed that the agglutinating action of this substance could be shown not only for sensitized and "alexinized" red blood cells, but also for similarly treated bacteria, and that conglutinins were present not only in bovine serum, but in that of goats, sheep, antelopes, and a number of other herbivores, but absent in cats, dogs, guinea pigs, and birds.

The body described by these workers as conglutinin is probably identical with a similar heat-stable serum component reported by Manwaring (55) and called by him "auxilysin."

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* Browning (14) had shown that horse alexin may be absorbed by sensitized beef cells without causing hemolysis.

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CHAPTER VIII

FURTHER FACTS ABOUT ALEXIN

Origin of Alexin. Buchner compared the action of alexin to that of an enzyme or ferment, and thought that the source of this substance might possibly be found in the white blood cells. This was suggested by the observation that bacteria were destroyed within the white blood cells, after phagocytosis. Hankin (1) maintained the leucocytic origin of alexin on the basis of the observation that increased bactericidal properties closely followed upon periods of leucocytosis.

After Hankin the problem occupied the attention of Denys and Havet (2), of Hahn (3), of Van de Velde (4), and others. This work was done before the complex nature of the bactericidal constituents of serum had been demonstrated and before the work of Schattenfroh had shown that the bactericidal substances extracted from leucocytes were quite distinct from the active elements of the serum.

No proof of the presence of alexin, either hemolytic or bactericidal, within the leucocyte or mononuclear cells has been brought by methods of extraction, and the apparently positive results reported by earlier observers are explained by the discovery of the heat-stable and non-reactivable bactericidal and hemolytic substances in extracts of such cells. The intracellular substances by which the digestion of ingested bacteria or blood cells is brought about may be entirely distinct from that of the serum antibodies and alexins. Neufeld (5) allowed leucocytes to take up highly sensitized red cells. Instead of undergoing prompt hemolysis, as they would if small amounts of alexin had been added, they were slowly broken up without hemolysis, fragments of hemoglobin remaining after complete morphological disintegration of the erythrocytes.

One of the basic premises of Metchnikoff's theory on the nature of alexin consists in the conception that alexin is not found in the circulating blood plasma, but appears only when there has been leucocytic injury.

Metchnikoff and his pupils (6, 7) in support of this idea, showed that in edema fluid produced by ligation — without cell injury — little or no alexin is found. This fact has been interpreted in favor of Metchnikoff's views, as has also the absence of alexin in the aqueous humor of the anterior chamber of the eye (6, 8). In this fluid no alexin is present under normal conditions, but if puncture is practiced, and the fluid taken after a period of three or four hours, alexin is found, probably, according to Metchnikoff's school, because of the coincident entrance of leucocytes into this space. It is conceivable, however, that an injury which is followed by the invasion of leucocytes is pretty sure to be followed by entrance of the fluid elements of the blood. Gengou (9) approached this problem by methods in which coag-

ulation was avoided. He took the blood through a paraffined cannula into tubes that had been coated with paraffin, and centrifugalized at low temperatures. This plasma and the material with which the experiments were done actually consisted of blood serum. Upon examining serum so obtained, he found practically no bactericidal action. As a result of this investigation he claimed to have demonstrated the truth of Metchnikoff's contention that the circulating blood plasma contains no alexin.

It has also been claimed by Walker (10) and by Henderson Smith (11), that, as serum stands upon the clot, it at first gains in alexin or complement contents, an occurrence which they attribute to the liberation of alexin from the leucocytes. This observation has not been universally confirmed.

The present point of view — based largely on the observation of intravascular phagocytosis and on the reaction of animals to injection with bacteria of the types amenable to bactericidal destruction, is strongly in favor of the presence of active alexin in the normal blood.

Cobra Lecithin. In investigations dealing with the hemolytic action of cobra venom it seemed at first as though a clue to the nature of alexin had been found. Flexner and Noguchi (12, 13) made the observation that cobra poison alone does not hemolyze the blood cells of cattle, goats, or sheep, if these cells are washed free of serum. This suggested that the serum of these animals contained some activating substance, and that the cells of other animals hemolyzed by venom, even when freed of serum, might contain an activating substance within themselves. The behavior of this activating substance was therefore similar to the action of complement, except that, as Calmette (14) showed, almost all sera were rendered more efficient for the activation of snake venom when heated to 65° C. Kyes (15, 16, 17) extracted the red blood cells of rabbits and other animals whose cells were hemolyzed by snake venom alone, and with these extracts could activate the venom against ox, goat, and sheep corpuscles, cells which were not ordinarily hemolyzed by the venom. Activation of the venom with extracts of the ox, goat, or sheep corpuscles was not possible. He concluded from this that the blood cells of the rabbit, dog, guinea pig, and man possessed an "endocomplement" for the snake venom.

This "endocomplement" was ether-soluble, and lecithin, added to the corpuscles and the snake venom, brought about a rapid hemolysis. This seemed to explain both why heated serum could activate the venom, and why some varieties of blood cells could be hemolyzed with serum, since lecithin is widely distributed in the fluids and cells. Von Dungern and Coca (18) refuted the "endocomplement" conception by showing that the cobra venom contains a lipoid-splitting ferment which acts upon the lecithin, liberating substances from it which hemolyze.

In further attempts to define alexin chemically, von Liebermann (19) and Noguchi (20) suggested that the alexin may consist of a combination of soaps and proteins. Noguchi (20) showed that the hemolytic organ-extracts described by various observers were soaps, a possibility which had been previously considered by Sachs and Kyes (16). Subsequent studies by other investigators (21, 22, 23) have shown that the hemolytic action of fats and soaps is not destroyed by heating and has no relationship with that of alexin.

Alexin Action and Concentration. Experiments by Kiss (24) have shown that the action of complement is quantitatively increased by concentration. For instance: "0.05 complement is sufficient to hemolyze completely a definite quantity of sensitized blood cells if the experiment is done in a total volume of 5 cc. 0.02 cc. of complement gives absolutely no hemolysis in a similar volume. When, however, the total volume is reduced to 2.5 cc., then 0.02 cc. of the complement begins to act, and it produces complete hemolysis if the total volume is reduced to 1.25." If sufficiently concentrated, a very small amount of complement can act upon an extremely large amount of red blood cells.

Filtration of Alexin. In connection with filtration experiments carried out for the purpose of demonstrating multiplicity of alexin, Muir and Browning (25) found that if alexin is rendered inactive by the addition of hypertonic salt solution up to 5 per cent, it passes through the filters and the filtrate can be reactivated by dilution to isotonicity. Sensitizer always passes through, but under ordinary conditions filtration through Chamberland or Berkefeld filters removes considerable proportions of alexin.

Inactivation of Alexin. It is one of the basic attributes of alexin that 56° C. for one-half hour destroys it. The process by which this moderate amount of heating inactivates is not definitely understood. One of the earlier ideas was that heating produced diminished dispersion of the proteins in which the complement was contained. The physical interpretation of inactivation was encouraged by the fact that other physical methods, such as prolonged shaking, allowing to stand without heat, and the addition of neutral salts, could lead to permanent or temporary inactivation of the alexin. Traube (26) determined a fall of surface tension as serum was heated to 56° C. He also showed that gradual restoration of the surface tension takes place as the serum is allowed to stand. It is not inconceivable, therefore, that the inactivation of complement by heat may depend upon an alteration of its colloidal state, which, if not carried too far, may be reversible and followed by gradual dispersion as the serum is kept twenty-four hours. On the same grounds the gradual deterioration of complement on standing may be compared to the slow settling out of colloidal suspensions which eventually results in spontaneous precipitation.

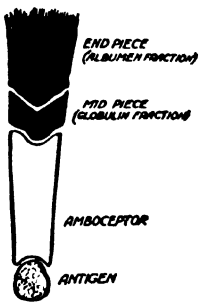
Effects of Salt Concentration on Alexin. Nolf (27) and von Lingelsheim (28) have shown that increasing the salt contents of serum (KNO_3, NaCl, K_2HPO_3, etc.) progressively diminished its bactericidal power. Hektoen and Ruediger (29) concluded that the action of the salts in such cases is exerted upon the alexin or complement and not upon the heat-stable sensitizers. The manner in which such salt-inactivation is brought about is obscure. There is no visible precipitation from serum after the addition of salts sufficient in quantity to weaken its action.

There is temporary inactivation which, at the same time, renders the complement filterable, facts from which we can only surmise some physical alteration.

Inactivation of the complement also follows the removal of salts, but here the process is accompanied by precipitation of the serum globulins.

Action of Acid and Alkali. Any considerable amount of acid or alkali added to fresh serum permanently removes alexin function. Brooks (30) determined that hydrogen ion concentrations high enough to transform serum proteins beyond the iso-electric point permanently inactivate it. Coulter (31) studied the activity of alexin at various hydrogen ion concentrations, both with and without the influence of heat. He found that the inactivation which complement undergoes when heated in distilled water dilutions is closely related to the properties of the euglobulin fraction, since the destruction is least at the reaction at which euglobulin is least soluble.

Alexin "Splitting." Buchner and Orthenberger (32) had been the first to show that bactericidal action was inhibited when salts were removed from the medium. Ferrata (33) found that the absence of salts exerted no effect upon the mechanism of sensitization. It was a natural inference, therefore, that the failure of hemolysis in salt-free media must be attributed to loss of complement function. Ferrata removed the salts from his sera by dialyzing against distilled water. In this process there is a precipitation of the globulins while the albumins remain in solution. The former may be redissolved in normal salt solution and the latter rendered isotonic by the addition of concentrated salt. Neither of these fractions alone would hemolyze sensitized red cells; both together exerted the original alexin function.



CONCEPTION OF COMPLEMENT-SPLITTING AS FIRST SUGGESTED BY BRAND

Brand (34) found both fractions equally thermolabile. The globulin sediment, after being redissolved in salt solution, cannot be preserved in an active condition for more than a few hours. Preserved in distilled water as sediment, it retains its activity for several days. Michaelis and Skwirsky (35) have since shown that the globulin fraction, thermolabile when free, is unaffected by a temperature of 56° C. after it has become attached to sensitized cells. The globulin fraction may attach directly to antigen-antibody complexes, but the albumin fraction cannot be bound in this way unless the globulin fraction has been previously attached. For this reason Brand referred to the former as the "end-piece" and the globulin sediment as the "mid-piece." It is possible, therefore, to treat sensitized cells with mid-piece in such a way that they are thereafter susceptible to hemolysis by the end-piece alone.

Hecker (21) confirmed the rapid deterioration of the mid-piece and showed that this does not imply destruction. For when such apparently inactive "mid-piece" was added separately to sensitized cells, and end-piece was subsequently allowed to act upon the complex, hemolysis resulted. This seems to show that the "mid-piece" undergoes a change on standing in salt solution which interferes with its ability to combine with sensitized cells only in the presence of "end-piece."

The combination of mid-piece with the sensitized cells can take place at a temperature of 0° C., while that of end-piece with the "persensitized" cells requires a higher temperature. Michaelis and Skwirsky (35), after determining that an acid reaction inhibits the hemolysis of sensitized blood cells, found that under such conditions "mid-piece" alone is bound, but that "end-piece" or the albumin fraction is left unbound.

Skwirsky (36) subsequently found that in the Wassermann reaction the syphilitic serum and antigen bind the mid-piece only. If the Wassermann reaction is strongly positive, active end-piece can be demonstrated in the supernatant fluid by the addition of persensitized cells. Bronfenbrenner and Noguchi do not believe that Skwirsky's experiments prove that end-piece is free in such "fixation" supernatant fluids.

That there is, after all, much similarity between the alexins of different animals is evident from the fact that, as Marks and others have shown, the end-piece of one animal may activate the mid-piece of another species. It appears also from experiments of Ritz and Sachs (37) that an animal may possess a mid-piece for certain sensitized cell complexes without possessing a corresponding end-piece. They found that the serum of mice contained a mid-piece but not an end-piece for the hemolysis of sensitized guinea pig corpuscles.

The most confusing feature of the problem lies in the quantitative relations. Thus Liefmann and Cohn (38) claim that in the presence of moderately sensitized cells no measurable amount of the globulin fraction is bound; yet, when both fractions are added to such cells, complete hemolysis results. In the presence of heavily sensitized cells (20 to 50 units) a small quantity only is removed. Nevertheless this fraction has had a demonstrable effect on the cells, since it has rendered them amenable to the action of the albumin fraction. The degree of sensitization must, therefore, be taken into consideration. It is curious that a slight excess of the globulin fraction may prevent complement action completely. In experiments cited by Marks (39, 39 a) it appears that the most effective complement is obtained when "mid-piece" and "end-piece" are added to the sensitized cells in proportions of 1 to 1. If the proportion of "mid-piece" is increased two or threefold over that of "end-piece," hemolysis is inhibited. This is true only when the two fractions are simultaneously added. When the sensitized cells are exposed to the excessive quantity of the "mid-piece" separately, and "end-piece" added later, the effect is one of stronger hemolysis than when smaller amounts are used. Bronfenbrenner and Noguchi (40) have suggested that the entire active complement is contained in the albumin fraction. They hold that "complement-splitting" by dialysis or other methods is an inactivation of end-piece by change of reaction. The mid-piece activates, they believe, by reason of its amphoteric nature and consequently adjusts

any excessive acidity or alkalinity of the medium. Liefmann (41) has been unable to confirm these experiments.

Neufeld and Haendel (42) have had difficulty in thus separating the fractions. Because of the occasional difficulties, other means have been devised. Sachs and Altmann (43) employed precipitation of the sera with weak hydrochloric acid, $\frac{N}{300}$ to $\frac{N}{250}$. Liefmann has separated the components by precipitation with CO_2. In carrying out this method, Fraenkel (44) has found it advantageous to dilute the serum ten times with distilled water, then allowing the CO_2 to flow in at low temperatures.

Later investigations now offer evidence that the original two fractions, the globulin mid-piece, and the albumin end-piece can be still further subdivided. In 1925 Whitehead, Gordon, and Wormall (45) found that by treatment with zymin a serum can be deprived of its hemolytic alexin action without removing the albumin and globulin fraction. Complement activity can be restored to such a serum by the addition of a little guinea pig serum heated to 56° C. This "third" alexin component is, therefore, heat stable and is associated with the globulin fraction. The same workers (46) showed that hemolytic alexin can also be inactivated by the addition of ammonia or ammonium salts. A serum so inactivated can be reactivated with heated guinea pig serum. The ammonia inactivation was active at *p*H 8 and over and was not merely due to reaction, since inactivation at the same ranges with other alkalies could not be reversed.

Ammonia inactivated alexin is not identical with that inactivated by zymin, since in the latter case the substance destroyed (by the ammonia) was associated with the albumin fraction and zymin-serum plus ammonia serum were found to reactivate each other. In removal of the "fourth" component time and temperature must be considered. The process is complete at 37° C. in one and a half hours. Carried on longer than this it is no longer reactivable. The fourth component resists heating to 56° but is destroyed at 66° C.

Spontaneous Reactivation of Alexin. Heating to 56° C., the simplest method of inactivations was at one time regarded as an irreversible process. Gramenitski (47) has shown that this is not strictly true. His experiments were suggested by the fact, observed by Bach and Chodat (48), that certain oxydases and diastases may spontaneously regain some activity after inactivation by heat. His work with complement indicated a similar gradual return to an active condition after moderate heating.

Experiment 1. Complement 10 times diluted was heated to 56° C. for 7 minutes. It was then tested against sensitized beef blood at varying intervals:

Time after Heating at Which Test Was Made	Quantity of Hemoglobin Gone into Solution after			
	% 10 Min.	% 20 Min.	% 30 Min.	% 40 Min.
Immediately after heating . .	0	20	40	70
1½ hours	0	30	60	80
24 hours	20	70	80	100
48 hours	10	40	70	—

When heating was more prolonged, a similar regeneration was observed, though not as pronounced as in the one cited. The largest amount of restored complement seemed to be present after about twenty-four hours. After this gradual deterioration again ensued. Traube (49, 50) determined a fall of surface tension as serum was heated to 56° C., and further showed that a gradual restoration of the surface tension takes place as the serum is allowed to stand. It is not inconceivable, therefore, that the inactivation of complement by heat may depend upon an alteration of aggregation of the particles, which, if not carried too far, may be reversible.

Brooks (30, 51, 52, 52 a) confirmed the observations of Gramenitski. He showed that after partial thermoinactivation, alexin recovers part of its hemolytic power, the recovery taking place more rapidly at 37° than at 7°. This may restore at least one third of its hemolytic action.

Inactivation by Shaking. Closely related to the preceding is the discovery made by Jacoby and Schütze (53) that complement can be inactivated by shaking. This has been confirmed by Zeissler (54), Noguchi and Bronfenbrenner (55), Ritz (56), and others. The success of such experiments depends upon the concentration of the serum, and is best observed in a dilution of 1 part to 10 parts of salt solution. Under such conditions complete inactivation may be observed within 20 to 25 minutes. Between the inactivation of complement by heat and that which results from shaking, there are certain similarities which strengthen the opinion regarding the nature of heat inactivation. It would be important to determine whether or not the inactivation by shaking, like that produced by heat, is accompanied by a fall of surface tension.

The Photoinactivation of Alexin (30, 51, 52, 52 a). The radiation of fresh serum with ultraviolet light to a slight extent inactivates alexin action. In this the temperature at which the radiation is done plays an important role. Brooks determined the actual inactivation by light radiation quantitatively.

Alexin Fixation. Whenever antigen and antibody unite, the new complex possesses the power to fix alexin, a property entirely absent or slight in either of the two reacting components alone.

The specific part of such reactions lies in the union of antigen with antibody. The subsequent fixation of the alexin is non-specific in the sense that any complex of this kind will unite with the alexin, whether the sensitization is one of a cellular antigen or of an antigen like horse serum, etc., in solution.

The practical utilization of these facts was made possible by the work of Bordet and Gengou (57) in 1901. Their experiments may be best described by submitting the protocol of their first experiment.

An emulsion of a 24-hour slant of plague bacilli was used as antigen, heated antiplague horse serum represented the antibody, and fresh guinea pig serum was used as alexin. A series of tubes was then prepared as follows:

1. Alexin + plague bacilli + inactivated antiplague serum.
2. Alexin + plague bacilli + inactivated normal horse serum.
3. Alexin + inactivated antiplague serum.
4. Alexin + inactivated normal horse serum.
5. Plague bacilli + inactivated antiplague serum.
6. Plague bacilli and normal horse serum.

These mixtures were left together for 5 hours and, at the end of this time, sensitized rabbit corpuscles were added to each tube. The result showed hemolysis in all the tubes except "1," in which there were plague bacilli, antiplague serum, and alexin, and in tubes 5 and 6, which had contained no alexin from the beginning. Similar results were obtained with anthrax, typhoid, and proteus bacilli, and their respective antisera.

The practical possibilities of this method were, of course, immediately apparent. By the use of a known antigen specific sensitizers can be demonstrated and, *vice versa*, in the presence of a known antibody, the method will serve to identify the nature of a doubtful antigen. Thus bacterial differentiation could be carried out by adding to the suspected bacteria, in emulsion, a small quantity of a known antiserum and alexin, and determining whether or not the alexin had become fixed. Bordet and Gengou (57) utilized the method in support of their claim of the specific etiological importance of the bacillus isolated by them from whooping cough, showing that the serum of children suffering from this disease formed a specific alexin-fixing complex with the bacillus.

A year later Gengou (58) showed that complement or alexin fixation was not limited to the complexes of cellular antigens and their antibodies, but that the sera of animals immunized with dissolved proteins (animal sera, etc.), when brought together with their specific antigens, likewise formed combinations which fixed alexin. Thus egg white or dog serum, brought together with "anti-egg white" or "anti-dog" rabbit serum, respectively, fixed alexin, whereas neither the antigenic substances nor the antisera exerted such fixation alone. Moreschi confirmed Gengou's experiments, and Neisser and Sachs (59) applied this knowledge to the determination of proteins for forensic purposes.

Fixation of Alexin by Precipitates. Moreschi (60, 60 a) studied the antihemolytic properties possessed by the serum of a rabbit treated with normal goat serum. He found that such a serum had anticomplementary powers when it was added to a hemolytic system of ox blood sensitizer (obtained against ox blood from rabbits), and goat complement. With such a hemolytic system, however, there was anticomplementary action only against goat complement and not against rabbit or guinea pig complement. If,

however, he used a hemolytic system in which the amboceptor or hemolytic sensitizer employed was one obtained from a goat, the serum was anti-complementary for all complements which were used. Moreschi concluded from this that the anticomplementary action of the serum resulted from the reaction which took place as the consequence of union of the antibody in the anti-goat rabbit serum and goat protein, which was introduced into the tubes, in the first case as complement, and in the second as amboceptor. He proved his contention by obtaining universal anticomplementary action when he added a little normal goat serum to the tubes set up as above described. Similar findings were later recorded by Muir and Martin (61).

Gay, confirming Moreschi, recognized the relation of the conditions observed in the precipitin reaction. He noticed that inactivated hemolytic immune serum, left for some time in contact with its specific cells, and then separated from them by centrifugation, would often possess anti-alexinic properties. After such a serum had been freed from the cells by a short centrifugation, if it was again vigorously centrifugalized, a cloudy sediment appeared at the bottom of the tubes. If this sediment was removed, the serum lost alexin-fixing properties. He recognized that the precipitate formed in these tubes was a specific precipitate, due to the fact that insufficient washing of the cells gave rise to the formation of precipitin against the serum of the animal from which the cells had been taken, and subsequently insufficient washing of the cells employed in the tests furnished enough antigen to give a precipitin reaction in the tubes in which the inactivated hemolytic (and precipitating) serum was mixed with the cells.

Although specific precipitates will fix alexin, the formation of a visible precipitate is not necessary. The essential occurrence is the union of the antigen and its antibody and, as we shall see, this union may take place in certain quantitative zones without the actual formation of precipitate.* The following table illustrates this point, since it shows that complete alexin fixation can take place in tubes in which hardly any precipitate was observed and that partial alexin fixation may take place in tubes remaining entirely clear.

Sheep Serum + Antisheep Serum	Precipitate	Fixation of 0.5 cc. Guinea Pig Complement
0.5 cc. (1 : 20) + 0.5 cc.	+	Complete
0.5 cc. (1 : 50) 0.5 cc.	+ +	Complete
0.5 cc. (1 : 100) 0.5 cc.	+ + +	Complete
0.5 cc. (1 : 200) 0.5 cc.	+ + +	Complete
0.5 cc. (1 : 500) 0.5 cc.	+ + +	Complete
0.5 cc. (1 : 1000) 0.5 cc.	+	Complete
0.5 cc. (1 : 2000) 0.5 cc.	+	Complete
0.5 cc. (1 : 5000) 0.5 cc.	——	Partial
0.5 cc. (1 : 10,000) 0.5 cc.	——	Partial
0.5 cc. (1 : 20,000) 0.5 cc.	——	None

* See also zone phenomena in bacterial tests in Chapter VII.

It should also be remembered that, if to such a precipitate there is added an excess of the antigen, the precipitate may be partially dissolved, and this dissolved precipitate, as Gay (63) has shown, may possess fixation properties.

Dean (64) found that the alexin fixation in mixtures of antigen and antibody is quantitatively determined by the character of the precipitation. If antigen and antibody are mixed in such proportions that a heavy precipitate is rapidly formed, there is either a very slight or no fixation of alexin, and there is, consequently, no relationship whatever between the amount of the precipitate and the amount of alexin fixed. If, on the other hand, antigen and antibody are mixed in such proportions that the turbidity develops slowly, as a very fine and gradual flocculation, large amounts of alexin may be fixed. The relationship is such a delicate one that if, in a series of experiments, varying amounts of antigen are mixed with a constant amount of antibody, two definite points may be found, at one of which there is the heaviest precipitation, and at the other the most intense alexin fixation. In cases in which an excess of antigen produces incomplete precipitation, no alexin fixation can be expected. Dean believes that alexin fixation probably occurs during the earliest stages of the precipitation, when the particles of precipitate are small, and that after the stage at which turbidity is observed, very little alexin fixation takes place. Therefore, in order to obtain the maximum amount of alexin fixation, the alexin should be present in the tubes at the time when antigen meets antibody.

Non-specific Alexin Fixation. In all experiments which deal with alexin fixation by specific antigen-antibody complexes it is of importance to guard against the errors introduced by non-specific fixation. There are a number of factors which interfere with the action of alexin upon a sensitized antigen, either by non-specific absorption of the alexin itself or by producing physical conditions in the presence of which alexin cannot act.

Many tissue cells, in emulsion, will absorb alexin, and the same property may be possessed by tissue extracts. Von Dungern (65, 65 a) was the first to call attention to this, and Muir (62) showed that the stromata of hemolyzed red blood cells establish union with complement. It is not unlikely that this action of cells is due to their lipoidal contents. This suggestion was made by Landsteiner and von Eisler (66), who found that the petroleum-ether extracts of red blood cells possessed anticomplementary action which, to a limited extent, was specific toward the particular corpuscles from which the extracts had been made. Similar observations have been made by Noguchi (67). The protective action of the lipoidal extracts seems to depend upon cholesterin, and, since this is present in many tissues, their antihemolytic action is easily understood (68, 69).

Yeast cells and almost all bacteria in emulsion may possess alexin-fixing properties even though unsensitized. There seems to be no regularity in regard to this, but the fixation is usually sufficient to render the use of whole bacteria unreliable for specific fixation experiments. For this reason bacterial extracts must be used and careful quantitative controls made. Such fixation may be purely non-specific and due to adsorption comparable to that observed on the part of kaolin or quartz sand emulsions, or fixation by such bacterial emulsions may occur because of small amount of normal sensitizer present in the serum employed as alexin.

Since anticomplementary action has been noted in the case of a large number of extracts of such materials as wool, leather, etc., methods of alexin fixation, as applied to the forensic differentiation of blood, must be controlled with this in view (70, 71, 72).

Non-specific agencies which fix alexin may also be present in normal serum. Noguchi (67) found that serum may develop anticomplementary properties after heating. This anticomplementary action increased as the serum was heated to about 90° C. Above this temperature it is destroyed. He refers this property to the serum lipoids.

Neisser and Döring (73) noticed anti-alexinic properties of human sera which were destroyed on heating, and which they associate with disease of the kidneys, since they noted it in sera of uremic patients. Browning and McKenzie (74) observed similar heat-sensitive anti-alexinic action on the part of normal serum. Zinsser and Johnston (75) found that all normal sera will develop anti-alexinic properties on preservation at room temperature within a few days, and more slowly in the ice-chest. This anti-alexin is destroyed on heating to 56° C., and may be precipitated out with the globulins of the serum.

The Wassermann Reaction. Soon after Bordet and Gengou's experiments Wassermann and Bruck (76) showed that bacterial extracts could be successfully substituted for whole bacteria in these reactions. Citron (77), too, made similar observations, and indeed, we now know that the use of bacterial extracts is more suitable for these experiments than are emulsions of whole bacteria, since, as we have mentioned above, bacterial emulsions may often fix small amounts of complement of themselves (without specific sensitization), thereby confusing the results of the reaction.

On the basis of their experience with bacterial extracts Wassermann and Bruck (78) then determined that complement fixation could be carried out in tuberculosis when the various tuberculin preparations were used as antigen.*

* Complement fixation in tuberculosis is not yet on a practical or reliable basis. Claims of Besredka (79) for his new antigen promised a successful technique, but no extensive confirmation has followed up to the present time.

These investigations fell into the period during which active research upon the *Spirochaeta pallida* in syphilis was going on, and it occurred to Wassermann that the technique of complement or alexin fixation might be utilized in the diagnosis of syphilis. Together with Neisser and Bruck (80) he subjected this idea to experimental test. They used the syphilitic monkeys which were being observed in Neisser's clinic. Their method consisted in mixing inactivated serum from syphilis-inoculated monkeys with organ extracts, serum, etc., of syphilitic human beings, and adding a small amount of fresh guinea pig complement. After these materials had been together for a certain time, sensitized red blood cells were added. If the complement was bound during the first exposure, no hemolysis resulted and the reaction was regarded as positive. They drew the following conclusions:

1. Immune serum from monkeys, produced by treatment with syphilitic material, will sensitize syphilitic material from human beings or monkeys, so that an alexin-fixing complex is formed.
2. Complement fixation results only when the syphilitic immune serum of monkeys is added to similar material from men or monkeys, but not when added to organ extracts of normal men or monkeys.
3. Normal monkey serum has no such action.

They concluded that their results justified them in assuming a specific fixation due to specific antisyphilitic immune bodies in the blood of the treated monkeys. They excluded experimentally the possibility of fixation by a precipitin reaction resulting from the treatment of the monkeys with human material, by the fact that they obtained positive reactions only when the human material was obtained from luetic lesions.

The same authors with Schucht (81) extended their method to the diagnosis of syphilis in human beings. The same thing had been done shortly before their publication appeared by Detre (82) on a smaller material. It was soon shown that syphilis may be reliably diagnosed by complement fixation when extracts of the syphilitic organs, employed as antigen, are mixed with the inactivated serum of syphilitic individuals. It was incidentally shown by Wassermann and Plaut (83) that the reaction could be obtained not only with blood serum but also with spinal fluid in paralytic cases.

It was generally assumed, at this time, that the reaction in syphilis depended, as in the case of other infections, upon the presence in the syphilitic serum of specific antibodies. It was soon found, however, by Marie and Levaditi (84), Weygant, Kraus and Volk, Landsteiner, Müller, and Pötzl (85), and others that antigens perfectly capable of fixing complement in the presence of syphilitic serum could be produced from normal organs.

Wassermann's original method of antigen preparation consisted in using the liver or spleen of a congenitally syphilitic fetus. The organs were finely divided and emulsified in 4 to 6 parts of normal salt solution. Later the specific organ substances were extracted by Porges and Meier (86) in five times the volume of absolute alcohol for 24 hours. This alcoholic extract was evaporated *in vacuo* and the residue taken up in

salt solution and shaken until an even suspension resulted. Landsteiner, Porges and Meier, and others then introduced antigens produced by alcoholic extraction of normal organs of animals and of man. Landsteiner introduced the alcoholic extract of normal guinea pig, extracts of the heart and liver, and Weil and Braun (87) made use of extracts of normal human organs. These antigenic substances being alcohol soluble consist very largely of lipoidal extractives of the organ substances, and it has been found that such antigen contains sodium oleate, lecithin, and cholesterin. Indeed, Porges and Meier have claimed that a 1 per cent solution of commercial lecithin may be used with success. Browning and Cruikshank (88) have found further that the addition of small amounts of cholesterin to syphilitic antigen very largely increases its diagnostic value, and this idea has since been utilized by Sachs (89), Walker and Swift (90), and others. The fact that cholesterin added to alcoholic organ extracts increases the antigenic value of these for the Wassermann reaction is all the more curious inasmuch as cholesterin alone has practically no antigenic action.

It is thus perfectly plain that, in the first place, the antigen used for the Wassermann reaction is non-specific in a sense that it can be extracted from almost any variety of normal organs, and in the second place that, being alcohol soluble, it is, at least largely, lipoidal in nature. Moreover, non-specificity is conversely indicated by the fact that antigens made of *treponema pallidum* were found by Schereschewsky (91), Noguchi (92), Craig and Nichols (93), and others to be valueless.

In Wassermann reactions, where the lipoidal non-specific antigen is mixed with syphilitic serum, Jacobsthal (94) was the first to show, in the dark field, that visible precipitation occurs. This observation was the basis from which all subsequent flocculation reactions for syphilis are derived.

The first successful one of these was that of Meinicke (95, 95 a), who found that both negative and positive serum would precipitate over night in the incubator if four volumes of a lipoidal extract in distilled water were added. A diagnostically practical method was developed by Sachs and Georgi (96, 97), who modified their antigens by the addition of cholesterin and by a technique of preparing suspensions in such a manner that they were more easily flocculable than the ordinary Wassermann antigen. The result was that they obtained a lipoidal suspension which precipitated with Wassermann-positive sera but not with the Wassermann-negative ones.

Modifications of the Sachs-Georgi method have been numerous, the most important ones from the practical point of view being the quantitative sigma reaction of Dreyer and Ward (98), the Kahn reaction (99, 100), and the recent glycerol-cholesterol reaction of Hinton (101, 102).

For detailed descriptions of these methods we must refer the reader to any of the many available practical manuals.

In regard to the Wassermann body in the serum which reacts with the lipoid antigen, all that we know about it with certainty at the present time is that it is related, like true antibodies, with the globulins which are, according to most observers, quantitatively increased in syphilis both in the blood and in the spinal fluid. In some cases it has been found associated with the euglobulins (103); according to other workers it has been found in the pseudo-globulins. A few observers, like Forssman, believe that there need be no particular increase of globulins in syphilis and cast some doubt upon the globulins as the responsible substances.

In regard to the theory of the Wassermann reaction, there is still much difference of opinion and uncertainty.

Schmidt (104) has suggested that the reaction is a colloidal precipitation resulting from the union of serum globulins with the extract colloids in the antigen. In normal serum there is a colloidal balance between the albumins and the globulins, the albumin acting as a sort of protective colloid. In syphilitic serum the globulins are so changed, either quantitatively or in the degree of dispersion, that the albumin no longer exerts a sufficient protective action. Similar in principle are the views of Levaditi and Yamanouchi (105) and of Klausner (106), who showed and attempted to develop practically the fact that syphilitic sera are more easily precipitated by distilled water than are normal sera.

Bergel (107) has suggested the importance of specific lipase production as a cause of hemolysis, and intimates that the Wassermann reaction is due to fixation exerted by the products of the action of a specific lipase formed in the syphilitic body against "lues-lipoids." This theory is open to objections similar to those mentioned above, namely, that the antigen need not necessarily be a lues-lipoid, but may be derived from normal organs. Other theories have been brought forward by Bruck, Manwaring, and more recently by Rabinowitch (108). The data supporting most of these theories are too speculative to justify discussion at any length.

Another point of view has been brought forward in regard to the Wassermann reaction which is based upon the knowledge gained by investigations of the heterogenetic or Forssman antigens. Weil and Braun made the suggestion some time ago that if a true antibody reaction were at all involved in the Wassermann reaction, it could be conceived only as an auto-antibody production against materials derived from tissue disintegration incident to the disease.

The similarity of the heterogenetic antisera to the syphilitic serum is that both of them react, by precipitation and complement fixation, with alcoholic organ extracts chiefly composed, perhaps completely composed, of lipoids. The difference is that the heterogenetic antibodies react with the alcoholic extracts of certain animal species only,

that is, the heterogenetic antigen type or guinea pig group, while in syphilitic reactions the antigen can be obtained by alcoholic extraction from practically all animal species.

While the aqueous extracts of heterogenetic antigen organs or organ suspensions will produce the antibodies, and although such antibodies will react with the lipoidal alcoholic extracts of the same or similar organs, the lipoidal extracts will not by themselves produce antibodies. In other words, the lipoidal substance here, like the Wassermann antigen, is a haptene in the sense of Landsteiner, capable of uniting with antibody, but incapable of inciting it.

The question arises whether homologous lipoids in combination with a foreign protein like that of an infectious agent could lead to antibodies capable of uniting with a lipoid antigen. Basing their reasoning upon these considerations, Sachs, Klopstock, and Weil (109) carried out the following experiments:

They produced an alcoholic extract of rabbit kidneys, evaporated this to dryness, and took up the residue in a 1–10 dilution of swine serum. They allowed these mixtures to stand one night at room temperature and injected them intravenously into rabbits. Control rabbits were injected with swine serum alone and with rabbit kidney extract alone. They carried out Wassermann reactions on all the animals before treatment. The result was that all but one of eight animals treated with the combination became Wassermann positive, whereas none of those — seven in each series — treated with the separate materials developed positiveness.

They thus succeeded by appropriate treatment in which homologous lipoids were combined with a foreign protein in producing a typical Wassermann reaction in animals. From this they suggest that the "Wassermann" may be true antibody reaction of heterogenetic type in which homologous lipoidal substances resulting from tissue disintegration combined with some material derived from the infecting organisms can form a complete antigen leading to auto-antibody formation.

The fact that this process should be specific for syphilis and for a limited group of other infections including leprosy cannot be explained, but may possibly depend upon the peculiarly suitable nature of the protein or other constituents of the infectious agents themselves, for in Landsteiner's original studies it was found that swine serum was far superior in its properties of completing the lipoidal antigens than some other proteins. It is still unexplained why in the Wassermann reaction almost any lipoidal organ extract and many of the commercial lipoidal products, lecithin and cholesterin, are universally useful, though to different degrees. The problem is not solved by the investigation cited, but an experimentally approachable line of reasoning has been opened

Forensic Alexin Fixation Tests. Preliminary discussions of the principles underlying alexin or complement fixation have revealed that alexin is bound not only by sensitized cells, but also when an unformed protein antigen is mixed with its specific antiserum. For this reason it is possible by means of complement fixation to detect amounts of a foreign protein too small to be demonstrable by direct precipitation.

The method was, therefore, suggested by Neisser and Sachs (59) as an additional method for the forensic determination of unknown proteins.

The tests are carried out by mixing a dilution of unknown protein with given quantities of antiserum, adding small quantities of alexin (quantities determined best by previous alexin titration); these reagents are left together at 37.5° C., and then sensitized cells are added to determine whether or not the alexin has been bound.

The table taken directly from the article of Neisser and Sachs, *loc. cit.*, illustrates the method and indicates extreme delicacy.

0.1 human antiserum + 0.05 complement and variable amounts of different normal sera (brought to 1 cc. volume with salt solution); the mixtures kept 1 hour at room temperature. Then added 1 cc. 5 per cent washed beef blood + 0.0015 cc. amboceptor and left 1–2 hours at 37° C. The results are as follows:

Amounts of Normal Serum	Hemolysis on Addition of Serum of:							
	Man	Monkey	Rat	Pig	Goat	Rabbit	Ox	Horse
0.01	0	0						
0.001	0	0						
0.0001	0	moderate	com-	com-	com-	com-	com-	com-
0.00001	slight	complete	plete	plete	plete	plete	plete	plete
0.000001	complete	complete						
0	complete	complete						

In this test 0.00001 cc. of the normal human serum still gave almost complete complement fixation of 0.05 cc. of complement in the presence of 0.1 cc. of the antihuman serum. The table also shows that this reaction follows a general law of relative specificity so often noted in other reactions, namely, that of all the animals tested, the serum of monkeys alone gave reactions with the human antiserum; and this in quantities as small as 0.001 cc.

The forensic complement fixation reaction of Neisser and Sachs is both theoretically and practically valid. Its extensive use in many investigations for theoretical purposes has well established its reliability. However, it is more complicated and requires much more experimental training and care than does the simpler precipitin test.

We purposely omit discussion in this place of the diagnostic application of alexin fixation methods to various infectious diseases. As far as these are practically applicable, they will be considered in the section on clinical application of immunology.

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CHAPTER IX

AGGLUTINATION AND PRECIPITATION

I. Agglutination

General Considerations. If one adds to an even emulsion of bacteria a small amount of homologous immune serum, the microorganisms collect in small masses which at first give the suspension a granular appearance. Soon after this these gather in large flakes and sink to the bottom of the test tube. The speed and completeness with which this phenomenon occurs and the nature of the appearance of the flakes depend upon a variety of secondary circumstances which will be discussed. The phenomenon was described between the years 1889 and 1896 by Metchnikoff (1), Charrin and Roger (2), Isaeff and Ivanoff (3), Washburn (4), and others. The specificity of the reaction and its apparent independence of other serum phenomena, however, were not recognized until the specificity of agglutination was described by Gruber and Durham (5) in 1896.

Gruber and Durham were the first to propose the use of agglutination for purposes of bacterial differentiation. Thus an unknown organism can be subjected to agglutination in a known antiserum to determine whether or not it belongs to the same species as the one used in producing the serum.

By reversing the process, the reaction is equally important in the diagnosis of infectious disease, by agglutinating a known organism in dilutions of the serum of a patient suspected of being infected with bacteria of that species.

Of all the immunological reactions, agglutination is most easily carried out, and it has, therefore, taken an important place in the routine of all bacteriological laboratories. Detailed descriptions of the various methods need not be included in this discussion, and can be found in any of the technical manuals.

In the differentiation of bacteria by agglutination it is important to work with sera of high titer, in order to exclude error from group agglutination. There are also, independent of dissociation phenomena, differences in the agglutinability of individual species and strains which must be overcome by technical devices. Thus meningococcus agglutination is often unsuccessful and irregular at the ordinary incubator temperatures, and is therefore carried out by preference in a water bath at 56° C., and in this

group, as well as in that of the Brucella and some others, the method of agglutinin absorption must often be substituted for direct agglutination, since the essential process is the union with antigen which may or may not, according to circumstances, be followed by agglutination. Actual clumping may often be hastened in slowly agglutinating strains and brought about in strains that would otherwise not agglutinate except by special methods, if the tubes are actively shaken at the moment when the serum and bacterial suspension meet. This probably depends upon an immediate, even distribution of the available agglutinin throughout the suspended antigen.

In carrying out diagnostic tests with the serum of a patient against a known microorganism, as in the diagnosis of typhoid, undulant fevers, etc., it is of the greatest importance to know statistically the likelihood of normal agglutinins in human sera against the organism used. Both in typhoid fever and in undulant fever a dilution of 1 to 5 usually excludes normal agglutinins in previously uninfected and unvaccinated individuals. In using agglutination for the diagnosis of glanders in horses MacFaydean (6) has found that dilutions lower than 1 to 500 are without significance, since normal horses may agglutinate up to this point.

For convenience, standard suspensions have been devised by a number of workers, the ones best known being the carbolized typhoid suspensions of Ficker (7) and the formalinized suspensions of Dreyer (8). Such standard suspensions are particularly useful in comparative tests when it is desired to determine the rise and fall of agglutinin contents from day to day in the same individual.

Agglutination does not kill bacteria and does not depend upon the cooperation of alexin. Serum may be inactivated at 56° C. without interference with its power of agglutination. In such heated serum clumping takes place without bactericidal effects, and the agglutinated bacteria may continue to grow. In fact, it is possible to carry out the reaction in this way, and colon bacilli, typhoid bacilli, pneumococci, cholera spirilla, and other organisms, which ordinarily grow as free single cells, or at most, in chains of two or three, if kept in the incubator for ten to twelve hours together with homologous serum, may grow in long, delicate chains, like those of streptococci. This form of reaction has been especially studied by Pfaundler (9) (Thread Reaction).

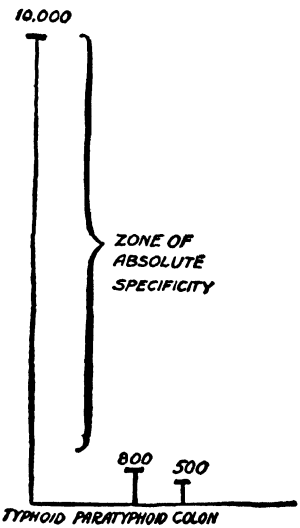
Group Agglutination. As in the cases of other immunological reactions the specificity of agglutination is subject to limitations. It has been found that, while immunization with any given species of bacteria gives rise to a marked increase of agglutinins for this species, there are formed at the same time, though to a lesser degree, agglutinins for bacteria of other species. This has been referred to as "group reaction," and the agglutinins appearing in such sera are spoken of as "*chief*" or "*major*" agglutinins and "*para*" or "*minor*" agglutinins. The table on the following page illustrates the overlapping group agglutination of the dysentery bacilli (Hiss).

As a rule, group agglutination corresponds closely to obvious morphological and biological relationships. Fundamentally, however, it must be referred to chemical similarities of antigens. Therefore, it is

not surprising that occasionally group agglutination seems entirely unrelated to morphological and cultural similarity.

	Anti-Shiga Serum	Anti-Flexner Serum	Anti-Y Serum
B. Shiga	1 to 20,000	1 to 100	1 to 100
B. Flexner	1 to 1200	1 to 3200	1 to 400
B. "Y"	1 to 100	1 to 600	1 to 6400

The diagnostic value of the specificity, however, is not affected by the phenomenon of group agglutination, since the action of minor agglutinins can be eliminated by sufficient dilution. Thus if we possess a typhoid-immune serum which agglutinates the typhoid bacillus in dilutions of 1 to 10,000, the paratyphoid bacillus 1 to 800 and the colon bacillus 1 to 500 (as in the figure), we may still utilize this serum for the identification of suspected typhoid cultures by employing dilutions above 1 to 800.



Diagrammatic Representation of Group Agglutination

The relation of agglutination to biologic relationship is not a simple problem, since individual strains even of the same species may vary considerably in agglutination by the same serum. Smith and Reagh (10) suggest the possibility that similar bacteria may acquire varying agglutinating properties by parasitism on different species of animals. This idea would explain the many serologic variants among culturally similar paratyphoid bacilli isolated from hogs, mice, rats, etc.

The animal species used for immunization influences the quantity and nature of the produced agglutinin considerably. In Pfeiffer's (11) experiments, a dog, a chicken, and a rabbit were immunized with the same strain of cholera spirilla. The sera obtained from these animals agglutinated this and other strains of cholera spirilla in an entirely irregular manner.

Agglutinin Absorption. Castellani (12) found that the immunization of an animal with two or more different species of bacteria results in the formation of agglutinins against all of them. Supposing that species A and B are used for treatment, agglutinins against both A and B are formed in quantity, depending upon the intensity of the treatment. If to the serum so produced an emulsion of A is added, agglutinin A only will be removed, while agglutinin B will remain in the serum almost

undiminished. An example of this is seen in the following protocol taken from Castellani's paper:

Titer of the Serum	Titer after Absorption with *B. typhi*	Titer after Absorption with *B. coli* "31"	Titer after Absorption with *B. coli* and *B. typhi*
B. typhi 4000 *B. coli* "31" 1000	*B. typhi* 0 *B. coli* "31" > 1000 < 300	*B. typhi* 4000 *B. coli* "31" 0	*B. typhi* 0 *B. coli* "31" 0

Immunization with a single organism, say *Bacillus typhosus*, may induce the formation of minor agglutinins for biologically similar organisms. In such cases, Castellani showed, absorption of the serum with the organism used for immunization takes out all of the agglutinins, major and minor. Conversely, however, absorption of such a serum with the species agglutinated by the minor agglutinins takes out these antibodies only, leaving the major substances intact. These relations are illustrated by the following protocols from Castellani's paper:

Serum of Rabbit No. 1 Immunized with *B. typhi* Only

Agglutination Titer of Serum	Titer after Absorption with *B. typhi*
B. typhi 5000 *B. coli* 600	*B. typhi* 0 *B. coli* 0

Serum of Rabbit No. 7 Immunized with *B. typhi* Only

Agglutination Titer of Serum	After Absorption with *B. typhi*	After Absorption with *B. coli*
B. typhi 10,000 (heavy clumps) *B. coli* 800	*B. typhi* 0 *B. coli* 0	*B. typhi* 10,000 (small clumps) *B. coli* 0

The method of agglutinin absorption is an excellent one for the demonstration of bacterial antigenic relationships and is regarded as more delicate than direct agglutination. It is in routine use in a great many procedures, such as in the typing of meningococci and the differentiation of the organisms causing undulant fever.

Complete absorption of specific agglutinin by a single exposure to homologous bacteria, however thickly emulsified, is not possible. It is always necessary to absorb repeatedly, and even then a minute trace

of agglutinin may eventually remain. Eisenberg and Volk (13) attribute this to the reversible nature of the union of agglutinogen with agglutinin.

Mechanism of Agglutination. Agglutination and precipitation are not dependent on the co-operation of alexin, and the agglutinating and precipitating power of a serum is not, therefore, destroyed by inactivation at 56° C. The thermal destruction of agglutinating and precipitating power varies between 70° and 80° C. according to a number of environmental conditions, just as in the case of antibodies in general, and as we have already stated in the section on the unitarian theory of antibodies, it is now generally accepted that agglutinin and precipitin are identical with the specific antibody concerned in other serum reactions.

In his original theory of agglutination and precipitation, Ehrlich regarded agglutinin and precipitin as separate antibodies and conceived their structure as twofold, including what he called a haptophore group which mediated specific union with the antigen, and a zymophore group which was responsible for the agglutinating effect. The two were inseparably united but the zymophore group was less heat stable than the haptophore and when a serum was heated to 70° the capacity for union with antigen was not destroyed but the agglutinating effect was eliminated. Such an altered agglutinin he spoke of as agglutinoid. In a subsequent section we will discuss the agglutinoid phenomenon more precisely.

The problem of agglutination was much clarified when Bordet discovered the importance of electrolytes in the reaction.

Bordet (14, 15) compared the formation of precipitates in bacterial emulsions to the precipitation of clay in water, and noted that the precipitation is "often controlled by such insignificant causes as the presence of salts." He performed the following experiment: Cholera spirilla, emulsified in salt solution, were treated with homologous immune serum and, after agglutination had taken place, the bacteria were thrown to the bottom by centrifugation and divided into two parts. One part was suspended in salt solution, and the other was washed, and then suspended in distilled water. The bacteria in the tube of salt solution rapidly agglutinated, while those in the distilled water remained suspended in even emulsion. If to these unagglutinated bacteria a small amount of sodium chloride was added, agglutination occurred.

The conclusions from this experiment are, first, that the bacteria could not agglutinate in the absence of salt even though they had been sensitized, and second, that the addition of salt to the sensitized suspension emulsions brought about immediate agglutination.

Bordet's conception was spoken of as the "*two phase*" theory, in that he showed the process of agglutination to consist of, first, an absorption

of the agglutinin by the bacteria, and second, an agglutination of the new complex by the salt. It is not the agglutinin which causes agglutination, but by union with the agglutinogen it forms a complex which is altered in suspension equilibrium, and is flocculable by the action of the electrolyte.

The conception of Bordet placed agglutination and precipitation into direct analogy with the phenomena of colloidal flocculation in general. As a matter of fact, bacteria in suspension have close resemblances to colloidal suspensions (16, 17, 17 a, 18). The organisms carry negative charges in neutral salt solution, wandering to the anode when subjected to cataphoresis, and this charge may be reversed by adding to the suspensions enough acid to carry the *p*H below the iso-electric point of the bacterial protein, that is, below approximately *p*H 4. Bacteria which have absorbed agglutinin, while they still usually migrate to the anode, do so more slowly or they may actually precipitate between the electrodes as though their charges had been diminished.

Studies on the influence of the action of electrolytes on bacterial suspensions have shown that bacteria which have absorbed agglutinin are flocculated by concentrations of salts which are too slight to affect the organisms in the absence of the specific serum. In this respect, bacterial suspensions show similarity to the behavior of emulsions of kaolin or mastic in the presence of weak solutions of gelatin.

The following protocols, adapted from the work of Neisser and Friedemann (17, 17 a), illustrate these conditions.

PROTOCOL CONSTRUCTED FROM THE TABLES OF NEISSER AND FRIEDEMANN (17, 17 a)

$\frac{n}{1}$ SOLUTION OF SALT	QUANTITY OF SALT SOL. WHICH BROUGHT ABOUT AGGLUTINATION OF 1 CC. OF *Normal Bacteria* IN EMULSION. 0 = NO AGGLUTINATION BY THE SALT SOLUTION	QUANTITY OF SALT SOL. WHICH AGGLUTINATED 1 CC. OF *Agglutinin Bacteria* IN EMULSION
NaCl	0	.025
$NaNO_3$	0	.025
Na_2SO_4	0	.025
$Cd(NO_3)_2$	.01	.001
$CuSO_4$	.0025	.0001
$CuCl_2$	.0025	.0005
$Pb(NO_3)_2$	.0025	.0001
$HgCl_2$	.0025	.0005

Just as the absorption of agglutinin by bacteria rendered these more precipitable by salts, so the addition of minute quantities of gelatin to mastic emulsions had a similar sensitizing effect upon these.

NaCl 10% Solution	1 cc. Mastic (1-10 Original Emulsion) Diluted to 3 cc.	1 cc. Mastic + .0001 cc. of a 2% Gelatin Solution, the Whole Diluted to 3 cc.
1.0	+ + +	+ + +
0.5	0	+ + +
0.25	0	+ + +
0.125	0	+ + +
0.05	0	0
0.025	0	0

An analysis of the mechanism of bacterial flocculation has been made by Northrop and DeKruif (19). Powis (20) had studied oil emulsions and had found that agglomeration of the oil particles occurred when the surface potentials between the drops of oil and the surrounding water were reduced below a critical value of about 30 millivolts. The stability of the emulsion was thus shown to depend upon the charges on the surfaces of the oil drops. Northrop noted that in the case of bacterial suspension this cannot be taken as the sole factor involved, because agglutination by immune serum could, on occasion, occur without marked change of the surface charges. For this reason, in his work with DeKruif, he took into account the cohesive force that acted to draw the particles together, and devised methods of measuring both this factor and the charges in relation to each other.

The surface charges, that is, electrokinetic charges, were measured by the rate of migration of the organisms in an electric field. The cohesive force was determined by covering a thick glass slide with a thin film of a heavy suspension of the bacteria, allowing it to dry, then heating to 60° for a few minutes so that the dry film adhered firmly to the glass. A heavy cover slip was similarly prepared. The cover slip was then suspended by means of a fine platinum wire from the lever of a de Noüy (21) surface tension apparatus. The slide was immersed in a dish containing the solution to be studied and the cover slip allowed to rest on it for one minute. The force required to pull the cover slip from the slide was then determined. If time intervals were allowed, between pressing down the cover slip and the measurement, the preliminary variations determined by the force with which the two surfaces had been pressed together became equalized and reliable readings were obtained. For details we refer to the original papers.

The results of Northrop and DeKruif (19) may be summarized briefly as follows: Salt added to bacterial suspension in water reduces both the surface charges which tend to keep the bacteria apart and the cohesive force which tends to draw them together. If dialyzed serum is added to a suspension of organisms in distilled water, the surface potential of the organisms gradually decreases, but not enough to cause agglutination. If salt solution is now added to the mixture, the potential decreases still further, and when this has dropped to the critical value of 13 millivolts,

agglutination occurs. The serum seems to prevent the salt from decreasing the cohesive force between the organisms, and the potential determines the agglutination when the salt becomes sufficiently concentrated to reduce this to approximately 13 millivolts.

Conversely, if the experiment is reversed and serum added to organisms in the presence of an amount of salt sufficient to produce this low potential, the serum gradually increases the cohesive force until it is greater than the repulsion due to the surface potential, and agglutination occurs.

Shibley (22, 23),* in studying the negative charges of various "normal" bacteria, showed that upon the addition of increasing quantities of immune serum this electrokinetic potential, as measured by movement in an electrical field, approaches the value of the charges upon particles of denatured globulin. At the same time, the cataphoretic iso-electric point, namely the reaction at which there is no surface charge and no movement in the field, shifts towards that of denatured serum globulin, approaching it most closely as sensitization — that is, the antibody on the cell surface — is increased.

Eagle (24), who has added considerably to the knowledge of these factors, summarizes the situation as follows: Agglutinating and precipitating antibodies represent immunologically altered serum globulin. The united antigen-antibody contains this globulin demonstrably, chemically, immunologically, and by the physical changes above described. In the case of the cellular antigens, the antibody is present as a film of specifically adsorbed protein, while in precipitation reactions it constitutes the bulk of the material precipitated. In both cases the originally water-soluble globulin has become denatured. Eagle suggests that, in the case of immune reactions, denaturization of the antibody-globulin is due to the fact that its specificity is determined by the hydrophilic groups. When these combine with the antigen, the hydrophobic groups face the water phase and determine the surface properties of the complex. When normal serum is similarly adsorbed, there are no groups with specific affinity to antigen and the molecules orient themselves so that the hydrophilic groups face the water.

Specific flocculation is, therefore, conceived by Eagle as follows: The antigen is covered with a film of globulin denatured by its union with the antigen. In the absence of salts the charge due to the ionization of this protein is enough to prevent flocculation. Electrolytes depress the surface charge below the critical value mentioned above and flocculation results.

About the formation of a layer of antibody globulin on the surface of the antigen there is little or no disagreement. Whether or not such a layer is to be regarded as a "film" of denatured protein, as Eagle assumes,

* See also Coulter, *loc. cit.*

is however still a question. Marrack (25) bases his objections to the "film" hypothesis partly on the following considerations. If serum globulin is used as antigen, one molecule of globulin could be covered by another in a layer about 10 Ä in thickness. In experiment, if globulin antigen is mixed with its antiserum, in optimum proportions for flocculation, each antigen molecule combines with about 4 antibody molecules. In the studies of Heidelberger and Kendall (26) 0.5 mg. Type III pneumococcus SSS combines with 27 mg. of antibody globulin. Even under conditions of the greatest surface exposure of this mass of antigen, the layer of globulin would have a thickness of 27 Ä. Marrack further suggests that, in the union of certain haptenes with antibody the antibody molecule must necessarily combine in "compact" form, since such haptenes offer no adequate surface for the spreading out of the globulin. His view conceives the antibody molecules as closely packed around the antigen attracted to it by its determinant groups. The polar groups on which the solubility of the globulin normally depends are thus brought into opposition with each other and attract each other instead of the water molecules.

The Agglutinoid Phenomenon. The conception of agglutinoids originated in the observation of the so-called "pro-agglutinoid zone." It was noticed in agglutination, as well as in other antigen-antibody reactions, that occasionally flocculation was absent in the tubes with the most concentrated serum but appeared in those in which the serum was moderately diluted. In other words, dilutions of 1–20 would not flocculate, but flocculation would be complete in tubes containing serum 1–80 and above. Eisenberg and Volk then showed that serum heated to 60° C. no longer agglutinated bacteria in the lower concentrations of 1–20 to 1–40 but might still agglutinate when more highly diluted. By moderate heating, in other words, they produced a "prozone" of agglutination or, in Ehrlich's view, they converted some of the agglutinins into "agglutinoids" which possessed an enhanced avidity for the antigen.

This pro-agglutinoid zone, which is of great importance in diagnostic work, was at first explained on the basis of the Ehrlich conception, described above. Before considering the modern views held to explain this phenomenon it may be well to mention a few of the observed facts. Natural prozones are relatively rare. Heating to 76° C. usually destroys all agglutinins. With certain sera, however, heating to 56° C. or slightly over, for anywhere from several hours to three minutes, according to the temperatures employed, produces a prozone. The agglutinating antibody in such heated sera is modified but its affinity for the bacteria is not destroyed. This is obvious from the fact that the treatment of bacteria with prozone sera, while it does not agglutinate, nevertheless prevents subsequent specific agglutination by the unheated serum. Shibley (27,

28) showed, moreover, that the cataphoretic behavior of bacteria treated with prozone sera closely resembles that of bacteria treated with unheated serum. It is apparent, therefore, that there is a union, in the prozone, between the organisms and an agglutinin modified by heat. It is probable, as Shibley states, that in the prozone there is a union between the organisms and a modified agglutinin, in the sense of the covering of the antigen with a film of this heat-modified globulin. And if this is the case, we must assume that in the lower dilutions of "prozone" serum, in which both modified and unmodified immune globulins are present together, the heat-modified protein is adsorbed in sufficient amounts to change the physical conditions that induce agglutination when only the unheated antibody is present. In other words, the prozone is due to the presence of heat-modified antibody on the surface of the bacteria. The exact nature of the modification of the globulin by heat in the production of agglutinoids has not yet been exactly determined. It is not impossible that the antibody protein is converted into what is spoken of as a protective colloid in the same way in which this occurs in the prevention of the precipitation of various colloids by fresh serum when minute amounts of heated serum are added.

Acid Agglutination. By "acid agglutination" is meant the spontaneous clumping of small particles of any kind, in suspension, in the presence of certain concentrations of acid. Michaelis (29, 30), Beniasch (31), and others have come to the conclusion that it is the concentration of hydrogen ions which is responsible for the agglutination. This explanation is also applicable to the agglutination often observed about the anode when bacteria are subjected in suspension to the action of a direct current. In such experiments the organisms after concentrating at this electrode often flocculate, and it is here, of course, that hydrogen ions are present in the greatest concentration. Schidorsky and Reim (32), Jaffe (33), and others have attempted to apply acid agglutination to the isolation and differentiation of bacteria, on the conception that different species are agglutinated by varying concentrations of hydrogen ions.

Much of the confusion brought about by such investigations has disappeared since it has become clear that acid agglutination is nothing more or less than a precipitation when the *p*H has been brought by the acid to a point corresponding to the iso-electric point of the bacterial protein, at which — being deprived of charge — it precipitates.

The Functional Importance of Agglutination in Infectious Disease. Since the property of specifically agglutinating bacteria is developed in the bodies of animals and man in the course of contact with bacteria during infection, it is natural to speculate concerning the possible significance of this phenomenon. Of course, it is quite possible that it is merely an incidental, inconsequential effect of reactions between antigen and antibody, and it has been so looked upon by a number of investi-

gators, such, for instance, as Metchnikoff (34), who says the part played by agglutination in acquired immunity is "merely accidental and subordinate." Salimbeni (35) goes so far as to state that bacterial agglutination does not take place within the animal body. His work was done with cholera spirilla. On the other hand, many observers have actually determined agglutination *in vivo*, the first observation of this kind reported by Sawtschenko and Melkich (36), who found clumps of the spirochetes of relapsing fever in the blood of infected patients. Also, in natural immunity, such as that, for instance, of pigeons against pneumococci, Keyes and others have seen clumps of bacteria in the capillaries of the liver and spleen. Bull (37, 37 a, 38) studied the matter in more detail and found that typhoid bacilli in the circulating blood of normal rabbits are rapidly clumped, removed from the blood stream, and accumulate in the organs, where they are taken up by polymorphonuclear leucocytes. This process is particularly active in the liver and spleen. In actively immunized animals, this is still more noticeable, both for the organisms mentioned, as well as for pneumococci, staphylococci, Shiga dysentery bacilli, and some other organisms. Bull's observation in regard to the action of pneumococcus immune serum, in pneumococcus infected rabbits, is particularly interesting since he found that the injection of sufficient amounts of anti-pneumococcus serum into septicemic rabbits resulted in a rapid clumping of the pneumococci in the blood stream.

It is apparent from this work that the clumping of bacteria in the body of the infected animal has definite protective functions which consist in preliminary concentration of the bacteria in the capillaries of various organs where phagocytosis is thereby facilitated.

Agglutination and Bacterial Dissociants. There have been a good many peculiarities in agglutination reactions for which it was difficult to account until the development of the knowledge of bacterial dissociation had clarified the antigenic relationships between rough and smooth bacteria and had demonstrated that in motile bacteria the nature of the antigen was influenced by the flagellar substances.

Spontaneous Agglutination. Spontaneous agglutination occurs in a variety of organisms and is a disturbing factor in practical work. It is particularly common in cultures of hemolytic streptococci, tubercle bacilli, diphtheria and diphtheroid organisms. It is not easily explained, depending upon an apparent tendency of the organisms to stick together after cleavage and to form clumps when they are brought into accidental contact in suspension. This tendency is apparently an inherent property of the bacterial protein which affects its suspension stability. Stevens (39) noticed that temperature affects this phenomenon and found that spontaneously agglutinating strains of streptococci might become diffuse and less granular when grown at room temperature. Shibley (40) has given

the matter considerable attention in connection with streptococci and concludes that it is due to a bacterial cohesive force higher than that usually found when bacteria are suspended in normal salt solution. Many of his strains could be made diffuse by growth at room temperature, reduction of salt contents in the suspension liquid and at a buffered reaction of *p*H 7 to 8.

Mueller, in our laboratory, has devised a useful method for overcoming the difficulties of spontaneous agglutination in hemolytic streptococcus cultures which depends probably upon principles similar to those suggested by Shibley, or may have something to do with a change of viscosity conditions. Mueller adds 0.2 cc. of normal horse serum to 10 cc. of the broth in which streptococci are cultivated. When the cultures have developed, although they may appear clumped in some tubes, shaking removes these clumps and the organisms appear in even suspension. It is always important to consider and adjust reactions, buffering excessive acidity. Agglutinations done with the antigen so prepared have been satisfactory in a large series of cases, although it is always necessary to set up the saline controls with similar additions of horse serum.

Hemagglutination. In their studies on hemolysis Bordet and others had noticed the frequent agglutination of red blood cells in the sera of animals treated with such cells after the hemolytic property had been destroyed by heating to 56° C. Such hemagglutination is analogous to the agglutination of bacteria by serum. Like the bacterial agglutinins, the hemagglutinins are relatively thermostable and are best observed after the sera are inactivated. Otherwise hemolysis will obscure agglutination. The hemagglutinins thus produced are specific, acting only upon cells which are used in their production. Certain sera may normally contain hemagglutinins for the blood cells of animals of another species. An illustration of this is the hemolytic and hemagglutinating property of normal goat serum for rabbit cells. Such normal hemolytic and hemagglutinating properties for the cells of other animals render the sera toxic for these animals. Within individual animal species there are isolysins and isoagglutinins analogous to those in human blood. (See chapter on Isoantibodies.)

II. Precipitation

General Considerations. In the early studies of agglutination many experiments were done to determine whether the development of agglutinins could be produced by the injection of bacterial extracts. It was an obvious thought to add some of the bacterial extracts instead of suspensions of whole bacteria to immune sera. Rudolf Kraus (41) was the first to perform this experiment. He was working with broth filtrates of *Bacillus pestis* and of the cholera spirillum, and found that when he mixed the clear filtrates of such cultures with their respective antisera, the mixtures would become turbid and subsequently develop flocculent precipitates. He named the reaction the "precipitin reaction" and spoke of the responsible antibodies as "precipitins." The reaction was found to be specific; the cholera serum gave no precipitate with the plague extract and *vice versa*, and Kraus, after extending his observations

to other bacteria, pointed out the practical possibilities of the reaction.

Though the first observations were made with bacterial filtrates and antibacterial sera, it was soon discovered that specific precipitins were produced whenever animals were treated with any antigenic substances. Thus Tschistovitch (42), in 1899, found that the blood serum of rabbits immunized with eel-serum gave specific precipitates when mixed with eel-serum, and Bordet (43) obtained analogous results by treating rabbits with defibrinated chicken blood and with milk.

There is nothing to be said about the precipitin-inducing antigen that has not been said about antigens in general in the special section devoted to this subject. Indeed, a very large part of the knowledge we possess about antigens has been developed as a result of precipitin studies.

Precipitation is limited in its specificity by group reactions. This fact does not in any way interfere with the practical value of the reaction because elimination of the secondary group reactions, which in agglutination is obtained by dilution of the antiserum, can here be obtained, as Kraus (44) pointed out, by diminishing the quantity of the undiluted precipitating serum added to the bacterial filtrates.

An illustration of such an elimination of "partial" or "minor" precipitins by diminution of the amount of the homologous anti-serum is given in the following table taken from the work of Norris (45):

ANTICOLI RABBIT SERUM

TABLE III

The precipitating action of the anticoli rabbit serum upon its corresponding filtrates and upon filtrates of B. N° 1 (hog cholera) and *B. typhosus*.

Coli Filtrate	Anticoli Serum	
0.5 cc.	0.05	Cloudiness in all tubes in 1 hour at 37.5° C. which increases rapidly. Six hours well-marked precipitation — most copious in tube containing 0.25 serum. Fluid in all tubes becomes clear.
0.5 cc.	0.10	
0.5 cc.	0.15	
0.5 cc.	0.25	

B. N° 1 Filtrate	Anticoli Serum	
0.5 cc.	0.10	At 6 hours a slight precipitate in the form of fine granules appears on the sides of the tubes. After 24 hours the precipitate in the tube containing 0.25 cc. serum compares in amount to that formed in the homologous filtrate with 0.05 cc. of serum.
0.5 cc.	0.25	

The practical value of the precipitin reaction, however, has not been in the special field of bacteriology.

Precipitins against coagulable proteins, egg albumen, animal sera, etc., are much more easily produced than are bacterial precipitins, and three

intravenous injections of from 2 to 5 cc. of the protein at 5- or 6-day intervals usually give rise to the formation of potent precipitins. When a small quantity of the serum of such an animal, taken 9 or 10 days after the third injection, is mixed with an equal quantity of a dilution of the antigen, turbidity and rapid flocculation result. For reasons which will be discussed below, precipitin reactions are carried out with undiluted or slightly diluted antiserum against increasing dilutions of the antigen. Thus, in testing the precipitating potency of, let us say, the serum of a rabbit immunized with sheep serum, we set up a series of tubes, each of which contains a constant amount of antiserum (precipitin), but progressively diminishing amounts of antigen in the same volume — *i.e.*, in dilution with isotonic salt solution.

Antisheep Serum from Rabbit		Sheep Serum 0.5 cc. of Following Dilutions:		Precipitation
0.5 cc.	+	1 : 10	=	±
0.5 cc.	+	1 : 100	=	+ + +
0.5 cc.	+	1 : 500	=	+ + +
0.5 cc.	+	1 : 1000	=	+ +
0.5 cc.	+	1 : 5000	=	+
0.5 cc.	+	1 : 10,000	=	—

The delicacy of the reaction is great, and sera have been obtained with which the specific antigen could be detected in dilutions as high as 1 to 100,000 (Uhlenhuth). A serum which will react with antigen in dilutions of 1 to 10,000 and 1 to 20,000 is not at all uncommon nor difficult to obtain. Apart from the advantage of the specificity of the reaction, therefore, this biological method of detecting proteins is more delicate than any of the known chemical methods; neither the Biuret nor Millon's reaction will exceed a delicacy of 1 to 10,000. By Complement or alexin-fixation, the delicacy of the biological reactions can be further enhanced.

The fact that precipitates fix alexin has been considered in a preceding chapter.

Mechanism of Precipitin Reaction. The study of agglutination and that of precipitation reveal, throughout, a close similarity between the two reactions, and in physical principles they are the same, although the one (agglutination) consists in the flocculation of large particles in suspension — the bacteria — while in the other the precipitation is one of smaller units — the antigen in solution.

A matter which has been puzzling in this connection is the fact that when one agglutinates bacteria, the serum can often be diluted many thousand times, whereas when doing precipitin reactions a dilution of 1–5 or 1–10 of the serum eliminates the reaction. The antigen in the

latter case can be diluted very highly and gives the precipitation reaction its differential value. The reason for this discrepancy can be explained on the basis of the following simple calculation.

Assuming that in both cases the mechanism is as we have described it in the chapter on agglutination, specific union of the antigen with an antibody coats the antigen with a layer of globulin from the immune serum. In consequence the antigenic particles behaved like globulin particles, their iso-electric point shifting to that of the globulin. The presence of electrolytes in the suspension reduces the potential but does not reduce the cohesive force to an equal degree, in consequence of which, when the potential falls below a critical value of about 12 to 14 millivolts, agglutination of the particles occurs.

If one considers the necessity of a coating of the particles by the antibody-carrying globulin of the serum, it is obvious that an immensely larger surface must be covered to precipitate a suspension of protein than is required for similar action upon a suspension of the considerably larger bacteria.

We can figure this out as follows (46): Let us suppose that two equivalent masses of antigen are subdivided, on the one hand into particles of ordinary bacterial size, on the other into particles of molecular size; and let us assume the average bacterial magnitude to be that of a sphere with a diameter of 5 μ, and that of the antigen particle to be that of a sphere with a diameter of 0.5 mμ. Thus, in millimeters:

Diameter bacterium = 0.005 mm.
Diameter antigen particle = 0.000,000,5 mm.

Then:

$$\text{The volume of the individual bacterium} = \frac{4}{3}\pi\left(5\,\frac{10^{-3}}{2}\right)^3$$

$$\text{The volume of the individual antigen particle} = \frac{4}{3}\pi\left(5\,\frac{10^{-7}}{2}\right)^3$$

and:

$$\text{Mass of bacterium} : \text{mass of particle} :: 10^{-9} : 10^{-21}$$

The amount of material composing a single bacterium, therefore, would be sufficient to form 10^{12} particles of the molecular magnitude. Now let us consider the surfaces exposed:

$$\text{The surface of a single bacterium of the specified size} = 4\pi\left(5\,\frac{10^{-3}}{2}\right)^2$$

$$\text{The surface of a protein particle of the specified size} = 4\pi\left(5\,\frac{10^{-7}}{2}\right)^2$$

and:

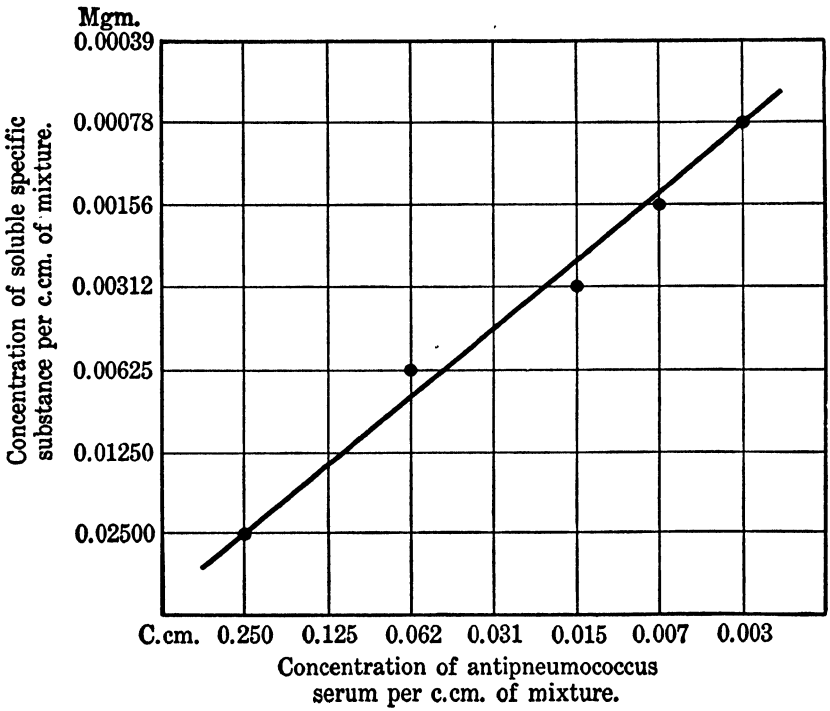
$$\text{Surface of protein particle} : \text{surface of bacterium} :: 10^{-14} : 10^{-6}$$

Thus the surface exposed by a single bacterium is 10^8 the surface of a single protein particle.

But in equivalent masses of material divided, respectively, into bacteria or into protein particles there would be 10^{12} as many particles as bacteria. Uniting this with the preceding statement regarding the surfaces, it becomes clear that the total surface exposed by a given mass of antigenic material divided into particles as large as bacteria would be to the total surface of same mass of material divided into protein particles as 1 is to 10^4, or as 1 is to 10,000.

It would therefore take 10,000 times more antibody globulin to coat the protein suspension than it would to coat the bacterial suspension.

This calculation brings the relationship of serum dilutions just about to where it usually is found experimentally.



Proportionality. Zone reactions are common in precipitin experiments, and it is obvious from what has been said that in the precipitin reaction the relative proportions in which antigen and antibody are mixed is of the greatest importance. This was most clearly demonstrated by Dean (47, 48) in 1911. With a horse serum anti-horse serum system Dean brought out quite clearly the principles of the proportionality which have been generally confirmed by subsequent workers. Parker (49), using our "residue" antigens, and Morgan (50), working

with pneumococcus SSS, were able to carry out similar tests in a manner quantitatively more satisfactory than was possible with serum antigens. The accompanying chart taken from Morgan's paper illustrates these conditions.

The curve represents the points of optimum precipitation with the varying amounts of antigen and antibody charted on the ordinate and abscissa respectively.

Dean's method of titrating by the optimal proportion method consists in making a preliminary rough test in which four dilutions of antiserum, 1 in 5, 1 in 10, 1 in 20, and 1 in 40, are tested against eleven dilutions of antigen ranging from 1 in 10 to 1 in 10,000. These mixtures provide an estimate of the approximate proportions in which the reagents give the most rapid and massive precipitate.

Precipitates, once formed, may be redissolved under a number of different conditions. If a precipitate is taken up in salt solution and resuspended, it may be dissolved by the addition of an excess of antigen. This is not always uniform. Marrack and Smith (51, 51 a), who examined the effects of diluting a reacting mixture of precipitin and antigen, found that the solubility of the precipitates formed by horse globulin and its antibody was about 0.35 mg. in 20 cc. and Felton (52), in a similar experiment with pneumococcus polysaccharide and antibody, found the solubility of the precipitate to be from 0.25 to 0.38 mg. of protein in 1 cc. Varying results by others with a number of different precipitating systems have shown that all precipitates are not equally soluble. Since, according to the experiments of Heidelberger and Kendall, the precipitin reaction must be regarded as reversible, it is not surprising that both reagents can be found in the supernatant fluid of series of mixtures. Dean (53) stated that at the optimum proportions there are practically none of the reacting substances in the supernatant fluid and Culbertson's (54) results are similar. It is probably true that at the optimum points there is a practically complete mutual precipitation, but at all other points there is every reason to believe that both reagents are measurably present in the supernatant fluid, although, of course, they may be in part united without necessarily being precipitated. Marrack and Smith have found that if an antigen added to a constant amount of antibody is gradually increased above the optimum proportions, the amount of globulin precipitated from the serum and the amount of antigen precipitated, both diminish.

No reaction has been as fertile as the precipitin phenomenon in throwing light on the quantitative conditions prevailing in antigen-antibody unions. In all precipitates the bulk of the material in the precipitate is derived from the antiserum. The relative amounts of the two constituents in individual precipitates depend, as we have seen, upon the pro-

portions of the two with which the reaction is done. Heidelberger and Kendall (26), working with Pneumococcus III antibody, purified by the Felton method, and pure solutions of specific Type III carbohydrate, found that as the soluble specific substance is added in increasing amounts to constant amounts of antibody, the smallest determinable amount of precipitate occurs when the relation between antibody and the carbohydrate is as 120/1 and, at the point of equilibrium, that is, when both substances are demonstrable in the supernatant fluid, the relation is about 60/1. The specific precipitate, then, is a mixture of varying proportions of the two components, depending on the relative amounts of the reagents. Heidelberger and Kendall, on the basis of a large series of quantitative experiments, believe that the entire reaction, from an excessive antibody to an excessive specific substance, may be expressed by three mass law equations in which the antibody can successively unite with increasing amounts of soluble specific substance at different levels, the proportions of SSS, combined with antibody, varying as 1 : 2 : 3.

Coexistence of Precipitin and Precipitinogen in Blood. An interesting phase of the study of precipitins is the occasional presence in the same serum of antigen and of precipitins which, though present side by side, do not unite. That such sera contain both antigen and antibody is shown by the fact that, though clear when taken, they will show precipitation not only when mixed with dilutions of the antigen, but also when added to homologous precipitating sera.*

This phenomenon has been noticed by Linossier and Lemoine (55), Eisenberg (56), Ascoli (57), and others, and by von Dungern (58). Gay and Rusk (59) have observed it in connection with the rapid method of precipitin production of Fornet and Müller, and have noted that such sera, although containing both antigen and precipitin, do not possess complement-fixing properties. According to Uhlenhuth and Weidanz (60), the antigen may persist in the sera of protein-immunized animals, in demonstrable amounts, as long as fifteen days. Two such sera, both containing antigen and precipitin, but neither of them precipitating by itself, will precipitate each other when mixed. For this reason Uhlenhuth has advised against the use of mixtures of precipitin sera for forensic tests. We are thus confronted by the paradoxical phenomenon of the presence in these sera, side by side, of an antigen and its homologous precipitin, incapable of reacting with each other.

Many attempts have been made to account for this. Eisenberg argues that both agglutinin and precipitin reactions take place according

* For instance, a rabbit was injected on three consecutive days with sheep serum. It was bled on the fifth day after the last injection. The serum was clear when taken, but a precipitate was formed when it was added to sheep serum and also when it was added to serum from another rabbit similarly treated and containing sheep serum precipitin.

to the laws of mass action. In consequence, in addition to the combined precipitin-antigen complex present in all mixtures of the two, there should also be present free dissociated fractions of each, in amounts dependent upon relative concentrations. This might explain conditions such as those described above. Zinsser and Young (61) have sought an explanation for the phenomenon in colloidal protection.

In spite of our own former prejudice in favor of the colloidal point of view, we are inclined to believe at present that the phenomenon is most likely to be explained on the original theory of Eisenberg. The studies of Heidelberger and his associates have furnished sound evidence for the conception of dissociation and equilibria in these reactions adequate to account for the phenomenon under discussion.

The Precipitin Reaction and Zoological and Botanical Species Relations. The extensive investigations of Nuttall (62) upon this subject have led to the development of a method for the determination of zoological species relationships. Nuttall carried out 16,000 precipitin tests with precipitating sera upon 900 specimens of blood which he obtained from various sources. He not only confirmed many of the accepted zoological classifications, but shed much light upon a number of disputed points. In working out the tests upon monkeys he found that the reactions carried out with antihuman serum become weaker as the species examined is farther removed from man zoologically. Thus as we read down the column from man to the *hapalidae* the precipitate becomes less and less in amount.

NUTTALL'S TESTS WITH ANTIHUMAN SERUM (63)

	ANTIHUMAN PRECIPITATING SERUM
TESTED AGAINST	PRECIPITATE
34 Specimens human blood	100% *
8 Simidiae, 3 species	100%
36 Cercopithecidae	92%
13 Cebidae	78%
4 Hapalidae	50%
2 Lemuridae	0

In another series he finds:

	ANTIHUMAN PRECIPITATING SERUM
TESTED AGAINST	PRECIPITATE
Man	100%
Chimpanzee (loose precip.)	130%
Gorilla	64%
Ourang	42%
Cynocephalus mormon	42%
Cynocephalus sphinx	29%
Ateles	29%

* The percentages refer to the volume of precipitate formed on standing for a given time, the amount formed by the antiserum with its specific antigen being taken as 100 per cent. Antigen dilutions correspond throughout.

Among the primates the highest figures with antihuman serum are given by the chimpanzee. Other bloods than those of the primates gave slight reactions or none whatever with the antihuman serum.

In addition to these results the relationships within the dog family, the horse family, and many other kinships similar to these were confirmed. In every case the precipitin reaction was consistent with the results of other methods of classification, and Nuttall's work is a valuable aid to zoologists in disputed questions of animal relationships.

Isoprecipitins, that is, precipitins resulting from the treatment of animals with blood of another individual of the same species, have been described by Schütze and others. They are not, however, regular in their appearance, nor are they very potent when obtained.

The reaction is equally applicable to vegetable proteins, and analogous investigations on the interrelationship of different varieties of wheat have been carried out by Magnus (64).

The Forensic Use of the Precipitin Reaction. The methods of performing precipitin tests for forensic or other purposes is extremely simple. Nevertheless, there are a number of theoretical considerations which we must take up in order to make clear the limitations of accuracy and conditions of control which are involved in these reactions.

From our discussion of the nature of precipitinogen it follows that blood stains, etc., on linen or articles of any kind will be suitable for precipitin tests even after they have been exposed for considerable periods to unfavorable conditions, that is, an environment in which they are subjected to exposure to light, moderate heat, or drying. Thus blood spots, etc., if kept dry and in the dark, may give positive reactions after years, as experiments by Uhlenhuth have shown. Meyer (65) claims even to have obtained a precipitation with extracts of the material of mummies. One of his specimens was a mummy dating back to the first Egyptian Empire (5000 years), the other about 2000 years old. Pieces of the leg and neck muscles of these specimens were chopped up finely, extracted for 24 hours with salt solution, then filtered until clear. With antihuman serum they gave turbidity after one hour at 37.5° C.

Under conditions of putrefaction, the precipitinogen is more rapidly destroyed, though blood putrefies with surprising slowness, even if the conditions of moisture, temperature, and reinoculation with putrefactive bacteria are constantly observed. Under such conditions a weak reaction may be obtained after as long as a month or six weeks.

In carrying out the tests with any material it is first necessary to get it into clear solution, a result which is best accomplished by soaking it in a small quantity of isotonic salt solution. Preliminary to this it is always necessary to scrape off a bit of the specimen and examine it microscopically to discover, if possible, whether blood cells, sperm, or other cellular constituents can be detected. The infusion in salt solution should be continued for several hours — if necessary for 12 to 24 hours. After the first few hours in the incubator the material should be placed at room or refrigerator temperature so that the yield in unchanged protein may not be diminished

by the action of bacterial growth. After extraction the solution may be filtered in order to clear it, but often centrifugation suffices. The concentration of antigen in such an extract is always an uncertainty, but may be determined with sufficient accuracy for practical purposes by shaking and observing the formation of a lasting foam. Protein solutions will show foam on shaking in dilutions as high as 1 to 1000, and if the original amount of salt solution is properly gauged to the amount of blood available, it is usually a simple matter to obtain a final concentration approximating 1 to 1000.*

The antiserum which is used should be of such a potency that preliminary titration with the specific antigen, diluted 1 to 1000, should give an almost immediate cloudiness at room temperature.

By testing this serum against a number of other varieties of protein — dog serum, beef serum, etc. — it must be determined that the precipitin in this case is strictly specific.

The reaction can be observed with greater delicacy if it is first set up by the method recommended by Fornet and Müller (66), which we may speak of as the "ring test." The antiserum is put into the tubes and the solution to be tested is allowed to flow slowly over this — as in Heller's nitric acid albumin test. At the line of contact between the two a fine white ring will rapidly appear, thickening and growing heavier as the preparation is allowed to stand. After taking the readings from such a test after an hour, it is well to shake up the tubes, set them away in the ice-chest, and again read the amount of precipitates formed in the various tubes the next morning. Since every test of this kind necessitates a number of controls, the following example will serve as an illustration:

FORENSIC BLOOD EXAMINATION

Material: Blood spot on trouser pocket, washed up in salt solution. Clear after paper filtration.

Antiserum: Rabbit treated with three intravenous injections, 2, 5, and 5 cc. of human serum at six-day intervals; bled on tenth day after last injection. This serum has been titrated against human serum and gives precipitation in dilutions up to one to ten thousand. With one to one thousand there is clouding which begins in three minutes and is very distinct in eight minutes, at room temperature.†

TEST

Tube 1.	Known human serum 1 to 1000	1.0 cc. +	Antiserum 0.2 cc.
Tube 2.	Unknown solution to be tested	1.0 cc. +	Antiserum 0.2 cc.
Tube 3.	Unknown solution to be tested	1.0 cc. +	Normal rabbit serum 0.2 cc.
Tube 4.	Salt solution	1.0 cc. +	Antiserum 0.2 cc.
Tube 5.	Unknown solution	1.0 cc. +	Salt solution 0.2 cc.

* If there is enough material, the amount of dissolved protein can be also approximately gauged by adding to a little of it a drop of acid, boiling, and observing the heaviness of the cloud which forms. A control test of a known dilution of the suspected variety of blood can be made at the same time and the heaviness of this cloud compared with that in the test solution. We believe that, in practice, several dilutions of the suspected material ranging from 1 to 100 up should be used if possible, for comparison with analogous dilutions of known human blood.

† A mixture of two specific antisera should never be used, since such sera may often precipitate each other for reasons that have been discussed.

In this test, if the original material was human blood, tubes 1 and 2 should show ring formation within 5 minutes, while the other tubes remain clear. In addition to these controls it is well to be sure that the test extract is neither strongly acid nor alkaline, and that the material from which it is extracted does not contain other substances, which can give precipitates by themselves when added to serum. This is especially necessary in the case of cloth fabrics, since such materials may contain colloidal dyestuffs or other extractable substances which might cause inhibition of precipitation.

Influence of Heating on Antigen (*Coctoprecipitins*). In the case of meat inspection, in which the precipitin test is useful in detecting admixtures of horse flesh, dog flesh, or other less desirable varieties of meat, in sausages, chopped meat, etc., it often happens that heating or smoking may vitiate the results of precipitin reactions. It is of importance, therefore, that one should know what the effects of heating (boiling) may be upon precipitinogen.

Obermeyer and Pick (67) found that precipitin produced with unchanged (native) beef serum did not react with heated beef serum, even if immunization was prolonged and a very potent serum was produced. On the other hand, when animals were immunized with beef serum which had been boiled for a short time ("Kurz aufgekocht") the precipitin so produced reacted, not only with native beef serum, but also precipitated the boiled serum and a whole row of split products which give no reaction to normal precipitin. The "coctoprecipitin" so produced, furthermore, was found by them to be specific, acting only upon beef protein or its derivatives.

Schmidt (68) states that heating serum protein to 70° C. for as long as 30 to 60 minutes alters its precipitability by "native precipitin" (precipitin produced by immunization with native unheated serum) only insofar as it diminishes the delicacy of the reaction by 10 to 30 per cent, and that heating to 90° C. for as long as an hour does not render it entirely non-precipitable, so that protein so treated may yet be detectable by ordinary specific precipitins produced by injections of unheated serum, though the delicacy of the reaction is lessened. Boiling, according to Schmidt, renders the antigen no longer precipitable by such "native precipitin," but, on the other hand, it does not seem to destroy its antigenic property of inciting precipitins on injection into animals.

He found that when he immunized rabbits with serum protein that had been heated at 70° C. for 30 minutes, the antiserum so obtained gave strong and practically useful reactions with its specific antigen even if this had been boiled. Since "native precipitin" gives weak reactions only with such a boiled protein, Schmidt recommends the use of the "70° precipitin" (produced by injections of heated serum) for tests in which a heated antigen is to be identified.

Very prolonged heating may so completely coagulate the antigen that none of it can be gotten into "solution" (suspension), and in such cases results can be obtained neither with the "native" nor with the "70° precipitin."

The following protocol is taken from the work of Schmidt.

TABLE I

(W. A. Schmidt (69))

SOLUTION OF	NATIVE PRECIPITIN	HEAT (70°) PRECIPITIN	HEAT-ALKALI-PRECIPITIN
Native serum	Strong reaction	Good reaction	0 (very slight turbidity)
70° serum (heated 30 min.) .	Good reaction	Strong reaction	Strong reaction
100° serum (heated 30 min.) .	0	Good reaction	Strong reaction
70° serum treated with NaOH (used to produce heat-alkali-precipitin)	0	0	Strong reaction
Boiled insoluble serum, brought into solution with NaOH .	0	0	Good reaction
Native serum treated with NaOH in the cold	0	0	Good reaction

In experiments carried out by the writer with Ostenberg (70) it was attempted to determine whether or not precipitins could be produced by injecting animals with protein that had been boiled, and if so what the action of these substances would be upon boiled proteins. Contrary to the results of Fornet and Müller, it was found that sera boiled for 3 to 5 minutes injected into rabbits induced precipitins which acted upon boiled proteins, but at the same time it was determined that the antibodies so produced were no longer strictly specific.

EXPERIMENTS ON COCTOPRECIPITIN. TABLE II (MARCH 23, 1913)

Cross titrations — dilutions of sera solution boiled 5 minutes, precipitated with antisera produced by injections with similarly boiled material.

The readings here indicated were taken by "ring" test at the end of 30 minutes.

DILUTION	BEEF SERUM VS. ANTI-BEEF PRECIPITIN	BEEF SERUM VS. ANTI-DOG PRECIPITIN	BEEF SERUM VS. ANTI-SHEEP PRECIPITIN	DOG SERUM VS. ANTI-DOG PRECIPITIN	DOG SERUM VS. ANTI-BEEF PRECIPITIN	DOG SERUM VS. ANTI-SHEEP PRECIPITIN	SHEEP SERUM VS. ANTI-SHEEP PRECIPITIN	SHEEP SERUM VS. ANTI-DOG PRECIPITIN	SHEEP SERUM VS. ANTI-BEEF PRECIPITIN
1 : 20	+	+	+	+ +	——	+	+ +	+ +	+
1 : 50	+ + +	+	+ + +	+ +	——	+ +	+ + +	+	+ + +
1 : 100	+ + +	+	+ +	+	——	+	+ +	+	+ +
1 : 500	+ +	—	+	+	——	+	+	——	——
1 : 1000	±	—	—	±	——	±	±	——	——
Controls of boiled serum alone *									
1 : 20	——			——		——			
1 : 50	——			——		——			
Serum control									

* These controls were necessitated by the fact that the boiled serum suspensions were themselves turbid and occasionally showed slight settling on standing.

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CHAPTER X

ISO-ANTIBODIES AND THE BLOOD GROUPS

HERETOFORE we have emphasized the fact of species specificity in immune reactions. To the uninitiated this might be taken to imply that no antigenic differences exist between the tissue components of individuals belonging to the same species. But a conclusion of this sort would be erroneous, for it has been found that various members of the same species possess antigens — particularly substances associated with the red blood cells — which are distinct from those found in similar tissues of other individuals. This class of antigens are referred to as iso-antigens and their corresponding antibodies are called iso-antibodies. The latter, just as the corresponding species specific antibodies, may occur either naturally or arise in response to artificial immunization.

The possibility that lytic antibodies might be formed within an animal against its own tissue cells occurred to Ehrlich and Morgenroth (1) early in their researches. Experimentally they approached the problem in the course of their work on hemolysins in goat's blood. They found that it was comparatively easy to produce hemolysins — iso-hemolysins, as they called them — in one goat by treatment with the erythrocytes of other goats. Although the blood serum of such an immunized goat was strongly hemolytic for the blood cells of the goat which had furnished the cells employed as antigens, as well as for the erythrocytes of certain other goats, though not for all, it was never active against the cells of the animal in which it was produced. Moreover, it was established that cells which were lysed by serum of this sort were capable of absorbing the antibody from it, while the corpuscles of the goat in which the serum was produced failed entirely to bind the hemolysin. This antibody, therefore, could not be regarded in any sense as an autolysin. Thus, although they found no evidence for the formation of antibodies reacting with the cells of the animal injected with its own tissues (autolysins), they did seem to demonstrate antigenic differentiation between corresponding cells of animals of the same species. Based upon these early observations, the problem of iso-antibodies in general aroused considerable attention among serologists because it revealed the possibility of an unexpectedly wide range of antigenic differences in the proteins of similar cells among the individuals of a single species of animal. Further investigations along these lines were soon reported.

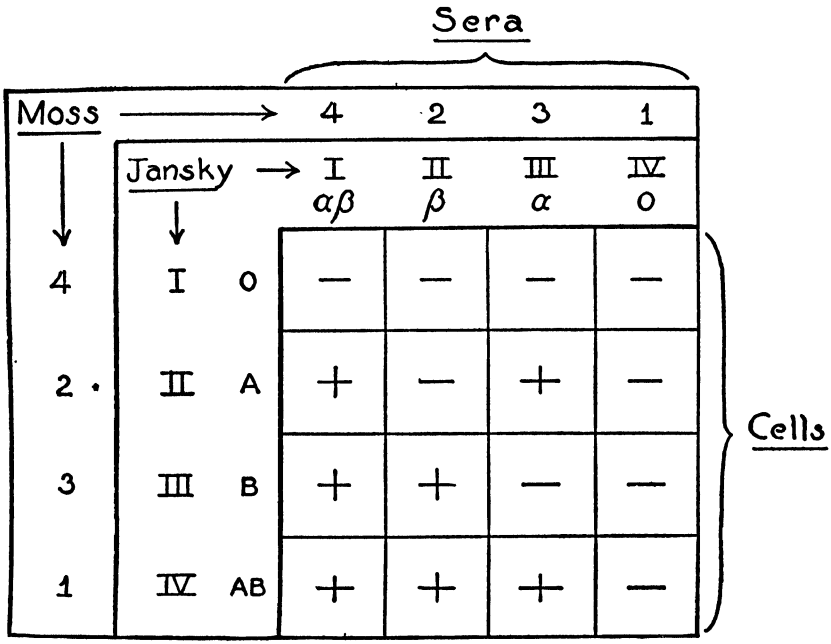
The results as far as they apply to the lower members of the animal kingdom will be summarized in a subsequent section. At the moment we will confine ourselves to a consideration of the iso-antibodies and their antigens which are found in man. The occurrence of such entities in human bloods was not definitely established until Landsteiner (2), in 1900, described normal iso-agglutinins and their corresponding agglutinogens in the red cells, and recognized them as due to physiological rather than pathological processes. Previously Maragliano (3) had reported that certain sera from patients with a variety of pathological conditions had the power of destroying human erythrocytes and hemoglobin, including the patients' own cells. This phenomenon, if it exists at all, is obviously different from that attributable to the action of isolysin. The same year in which Landsteiner's fundamental discovery was announced, Shattock (4) in England, also working with pathological sera, noted in some a tendency of the erythrocytes to settle out of the plasma and clump together. Because of this observation he has frequently been credited with priority in describing iso-agglutination in human blood. It now seems quite clear, however, that it was not true iso-agglutination which Shattock saw but so-called "pseudo-agglutination," which will be described below.

Landsteiner's (5) observation enabled him to divide 22 bloods from normal individuals which he examined into three groups on the basis of the agglutinative reactions that occurred when the serum and cells were separated and mixed with those from other bloods. A year later his pupil, Sturli, with von Decastello (6), added a fourth group which represented only a small percentage of those studied.

Landsteiner designated his three groups A, B, and C. The sera of group A agglutinated the corpuscles of group B but not of group C; the sera of group B agglutinated the corpuscles of A but not of C; the sera of group C agglutinated the corpuscles of both A and B. The sera of the fourth group described by Decastello and Sturli failed to agglutinate the corpuscles of Landsteiner's three groups, but its corpuscles were agglutinated by the sera of all of them.

From his own results, Landsteiner postulated the presence in various human sera of two iso-agglutinins and in the red cells of two corresponding antigens or agglutinogens. Decastello and Sturli by means of absorption experiments presented further evidence for the presence of two iso-agglutinins. Landsteiner assumed, and his assumption has been borne out by tens of thousands of determinations of blood groups in which only one or two exceptions have been noted, that an iso-agglutinin and its homologous agglutinogen could never exist together in the blood of the same individual. He considered their relationship to be reciprocal; *i.e.*, the absence of one implies the presence of the other.

In 1907 Hektoen (7) confirmed these findings of Landsteiner and his pupils, and, in the same year, Jansky (8, 9) made the first definite classification of the blood groups, assigning them the numbers I to IV. Moss (10), in 1910, unaware of Jansky's work, arranged them in a similar manner, but reversed the Jansky groups I and IV. The existence of these two systems has led to considerable confusion. For this reason von Dungern and Hirschfeld, among others, suggested that the groups be named O, A, B, and AB, according to the antigenic structures of the respective red blood cells. Their classification, which has come to be



known as the International nomenclature or that of Landsteiner, permits of no confusion and will, it is to be hoped, entirely supplant the use of the Moss and Jansky systems in clinics as it has in scientific publications. The table which we insert shows the correlation between the three nomenclatures. In the case of the Landsteiner classification the agglutinogens of the cells are designated by Roman letters and the iso-agglutinins with which they react by the corresponding Greek letters. Crosses, of course, indicate agglutination.

Since Landsteiner's fundamental observations, the grouping of vast numbers of individual bloods by means of the iso-agglutinins has shown beyond question that four main groups and only these exist among human beings. Nevertheless, certain results obtained by absorption

techniques have made it apparent that group A and AB bloods respectively may be further divided into two sub-groups (11, 12, 13). Thus it has been found that when the majority of group B sera are completely absorbed with certain group A (or group AB) corpuscles, these sera will still agglutinate red cells obtained from certain other individuals belonging to group A (or AB). Bloods containing corpuscles which react with such absorbed sera have been assigned to sub-group A_1 (or A_1B), whereas those possessing cells which fail to agglutinate in these sera are classified as group A_2 (or A_2B). These effects are in all probability due to the presence of two iso-agglutinins as Landsteiner and Witt (14) have shown. One of these is capable of reacting with both agglutinogen A_1 and A_2; the other reacts strongly with agglutinogen A_1 and only weakly or not at all with agglutinogen A_2. These iso-agglutinins are also found in nearly all O sera as well as in B sera.

The recognition of the A_1 and A_2 sub-groups in no way invalidates for practical purposes such as transfusion the arrangement of bloods into the classical four groups, since there is no evidence that agglutinogen A_1 can occur independently of A_2; *i.e.*, it does not represent an independent Mendelian factor. The principal significance of these sub-groups lies in their application to genetic studies and their relationship to medico-legal questions and to anthropological measurements.

Occasionally atypical or anomalous agglutinins have been noted. These appear to be of three kinds: (1) agglutinins for A_2 and O cells which occur in the sera of group A_1 or A_1B; (2) those found in A_2 or A_2B sera which react with cells of group A_1; (3) agglutinins for the cells of certain individuals irrespective of the group. These anomalous agglutinins are relatively rare, since, according to Landsteiner and Levine (15) and Thomsen (16), they appear in only about 3 per cent of bloods. It is the opinion of these authors that since their effect is noticeable in most cases only at about 20° C. or less, and disappears at 37° C., they represent potent "cold" agglutinins (see page 265). To these instances of irregular agglutinins may be added a remarkable case of the co-existence of B agglutinogen and an agglutinin which reacted with the erythrocytes of other individuals in the same group which has been reported by Ottenberg and Johnson (17).*

Inasmuch as these anomalous agglutinins do appear in some bloods, it is necessary before transfusion to carry out direct matching of the blood of donor and recipient, although the group of each has been previously determined by means of standard sera.

* For an extended account of anomalous agglutinins as well as the sub-groups of A the reader may consult Wiener's monograph (18), particularly pages 33 ff., pages 109 ff., and pages 141 ff.

The New Agglutinogens "M" and "N" of Landsteiner and Levine. In 1927 Landsteiner and Levine (19, 20, 21) described two hitherto unrecognized agglutinogens in human blood. These were termed M and N and were found to occur entirely independently of the agglutinogens A and B. The study of these new factors has since been vigorously pursued, but except in a single instance no homologous iso-agglutinins have been detected in human sera. The presence of these agglutinogens was demonstrated by producing immune sera in rabbits against the corpuscles of various individuals. Of course, agglutinins will not only be formed against the M and N factors, but also against the group agglutinogens A and B, if these be present, as well as against the human species specific factor (see chapter on Antigens). In practice it is customary to select as antigens red cells of group O containing either the M or the N factor. In this way the group agglutinogen is avoided. After a sufficiently potent serum is obtained, this is absorbed with cells of an antigenic pattern such as will remove the species specific agglutinin and leave the desired antibody against the M or N factor. For example, if an immune serum is produced against cells containing MO, it is absorbed with NO cells. Experiment has shown that anti-N sera are much more easily produced than those reacting with the M factor.

Landsteiner found that all human red cells contained either M or N or both together. On this basis three blood types are possible: M, N, and MN. Since no iso-agglutinins are normally present, these new agglutinogens have no importance, at least so far as it is now known, in considering suitable donors for transfusion. To us it seems at least theoretically possible that immune iso-agglutinins might develop following a transfusion of let us say M blood into an N individual, which, if the procedure were repeated using the same or a similar donor, could induce severe anaphylactic reactions. Like the sub-groups A_1 and A_2, the M, N, and MN types are of chief interest in that they extend the possibilities for application of genetic laws to problems of legal and anthropological interest. Additional agglutinogens have been detected in human blood erythrocytes by the method of producing immune sera and subjecting these to appropriate absorption. Of these one which they term the P factor has been described by Landsteiner and Levine (21) and two others designated H and G respectively have been reported by Shiff (22, 23). As yet their relative significance has not been fully assessed. For further details and bibliography the reader is referred to the excellent monograph of Wiener (18).

Technique of Determining the Blood Groups

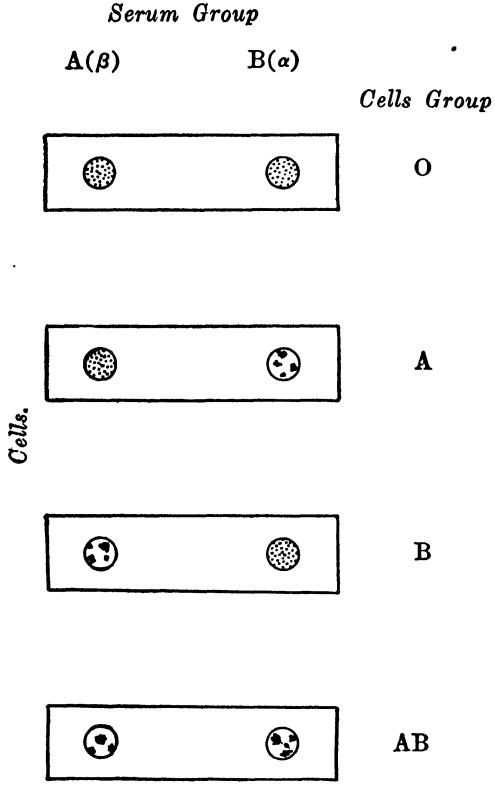
There are a number of methods by which an unknown blood may be assigned to one of the four classical groups of Landsteiner. They all

involve (1) the collection and preservation of two standard typing sera, containing respectively α and β agglutinin in high titer; (2) the preparation of red cell suspensions; and (3) the mixing of cells and typing sera under conditions which will give prompt agglutination, but which will also insure in so far as possible the absence of false reactions. Typing sera may be obtained by venipuncture from individuals known to belong to group A and group B. Very young and very old persons should not be selected as it is known that their sera are apt to be weak in agglutinin. Nor should the blood of diseased persons be used, since they frequently tend to give pseudo-reactions. After withdrawal the blood should be allowed to clot, the clot is rimmed with a glass rod and removed together with the cells by means of the centrifuge. It may possibly be of advantage to allow the tube containing the clotted blood to stand overnight in the ice-box before removing the serum, since in this manner certain of the "cold" or "auto-agglutinins" may be removed. The serum is collected sterilely and stored in the ice-box. Wiener recommends the avoidance of preservatives, but 0.5 per cent phenol may be employed or the dyes suggested by Rosenthal (24), which have been successfully used by the Boyds (25) in anthropological investigations in the field. The latter also carried as standards high titer anti-A and anti-B immune rabbit sera prepared by the method of Hooker and Anderson (26) because of their greater strength and speed of reaction. The Boyds (27) advise the use of immune sera not only for such field work but also in medico-legal cases as checks on the standard human typing sera. The titer of test sera may be determined by making serial dilutions and adding equal volumes of appropriate cell suspension. It is sufficient, however, to choose sera which give rapid and distinct macroscopic agglutination when 1 drop of the serum, diluted 1 to 10 with saline, is mixed on a glass slide with an equal quantity of cell suspension.

Suitable cell suspensions are prepared by catching about 5 drops of blood in 5 cc. of normal saline solution. This should give a 2–5 per cent suspension which is recommended by Landsteiner (28). The cells may be centrifuged and resuspended if excessive care is required, although this is not usually necessary. Freshly prepared cell suspensions should be employed, since anomalous reactions may occur if cells are preserved in saline for even 24 hours. If necessity drives, cells may be preserved for several weeks in the cold by the method of Rous and Turner (29).

Cell suspensions and the typing sera may be mixed either in small test tubes or on glass slides or cover slips. The tube method described by Landsteiner (28) is, on the whole, to be preferred, since it prevents evaporation which may give false clumping and it is possible to achieve a slight dilution of the serum by this method which greatly diminishes

the possibility of pseudo-agglutination. It consists in placing one drop each of unknown cell suspension, physiological salt solution, and standard serum in a tube of 7 mm. bore. After thorough mixing the tubes are allowed to stand at room temperature for one hour, when the final reading is taken, although in most cases visible reactions occur within a few minutes. Grossly apparent agglutination should be checked by microscopic examination under low power of a small drop of the contents of each tube transferred to a glass slide. A much more rapid effect may be secured by immediately centrifuging the tubes at high speed for three minutes and then shaking until resuspension of saline control cell suspensions and negative blood is obtained. The results are evident without recourse to the microscope.



The slide method consists in adding a drop of A serum and B serum respectively to a drop of the cell suspension on a slide. Adequate mixture is secured by rapidly rocking the slide back and forth. After 5–10 minutes a cover glass is dropped over each preparation and the result, if not immediately apparent to the naked eye, may be read under the microscope. No conclusion should be drawn until observation has been continued for at least 15 minutes. The results should be controlled and the standard sera checked by setting up simultaneously mixtures of known A and B cells. As a further precaution, the serum of the unknown blood may be added to the known A and B corpuscles.

A modification of the slide method has been described by Moss (30). It is probably to be preferred, since it prevents evaporation: a loopful of standard serum is mixed with the corpuscular suspension on a cover glass which is then inverted over a hollow ground slide with the usual liquid paraffin seal employed in hanging drop preparations. Final

readings under low power should be taken after one-half hour. The reactions which occur and which determine the group to which the unknown blood belongs are illustrated in the figure which follows:

When bloods are grouped for the purpose of selecting a suitable donor for transfusion, direct matching of the blood of the donor and recipient should always be carried out in addition to group determination by means of standard sera. This is essential because of the occasional presence of a-typical agglutinins and the possibility of unusually high agglutinin titers in the donor's serum. It also serves as a check on the grouping which has been previously determined. Cross matching may be performed by mixing 10 drops of the blood of donor and recipient respectively with 1 drop of 5 per cent sodium citrate solution in small tubes which are then immediately centrifuged. A drop of the supernatant plasma from the donor is then mixed with a drop of 2–5 per cent cell suspension prepared with the blood of the recipient on one end of a glass slide. On the other end is placed a similar mixture of the cells of the donor and the plasma of the recipient. One then proceeds in the same manner as described above for the use of the slide method in blood grouping. No reaction, of course, should occur in either preparation. A technique which eliminates the necessity of separating cells and plasma has been described by Coca, and recommended by Landsteiner (28), but that given here which we take from Wiener (18) appears preferable in those cases in which the group of donor and recipient are the same, since it removes the possibility of any confusion arising from the coexistence of the red cells of donor and recipient in the same preparation.

Reactions Which May Lead to Errors in Blood Grouping

Rouleaux Formation and Pseudo-agglutination. Certain bloods, particularly those obtained from persons suffering from a variety of pathological conditions, notably the infectious diseases, and also from pregnant women, are characterized by rapid sedimentation of the erythrocytes. This phenomenon is accompanied or conditioned by the arrangement of the red cells into packets or "rouleaux" having the appearance of stacked coins. This has been sometimes mistaken for agglutination, especially when it is marked and the rouleaux tend to clump together as in so-called pseudo-agglutination (26, 31). Confusion from this source can usually be avoided by slight dilution of the serum (1 : 3). Pseudo-agglutination, unlike "cold" and auto-agglutination, takes place at room temperature or at 37° C. and is apparently due to some unknown constituent of the serum which unlike the iso-agglutinin cannot be removed by absorption.

Cold Agglutinins. If a blood is kept at low temperatures (0–5° C.), clumping of the corpuscles frequently occurs. This effect is due to what

have been termed "cold agglutinins." Two varieties have been described by Landsteiner and his associates (31, 32): one shows a certain degree of specificity, while the other is entirely non-specific. The latter are referred to as auto-agglutinins. Both kinds may be absorbed from sera by treatment with suspensions of corpuscles and both are usually inactive at 20° C. and practically always at 37° C. In certain conditions they are abnormally increased, such as in paroxysmal hemoglobinuria and syphilitic cirrhosis of the liver. Abnormally potent cold agglutinins may act even at room temperature and thus lead to confusing results in the grouping of bloods. Certain of the specific cold agglutinins, as we have indicated above, may be involved in the anomalous reactions which are observed occasionally with bloods belonging to the sub-groups of A. This type of agglutination, unlike pseudo-agglutination, is not inhibited by slight dilution of the serum.

Anomalous agglutination sometimes occurs as a result of bacterial contamination — leading to the so-called Hübener-Thomsen phenomenon which has been studied exhaustively by Friedenreich (33). The recognition of this effect emphasizes the importance of employing only sterile reagents and fresh corpuscular suspensions in every instance.

The Necessity for Grouping of Bloods before Transfusion

Transfusions of the blood of a healthy individual or animal to patients weakened by disease were occasionally attempted at least since the sixteenth century.* Although occasionally they appeared successful, numerous fatal reactions were observed which served to discourage extended trial and rendered the procedure impractical. With Landsteiner's discovery of the human blood groups the way seemed opened for renewed experimentation with this form of therapy, which theoretically, at least, had always seemed indicated in cases where the red blood cells were reduced through trauma or other causes productive of anemia. Landsteiner himself at once recognized the possibility of applying his observations to the transfusion of blood, but at first little was done in this direction. With the advent of the World War, however, and the all too obvious necessity of in some way replenishing the blood lost as a result of severe wounds, transfusion based on compatible blood grouping became common practice. It was evident almost from the beginning that in most cases fatal reactions following transfusion could be attributed to *in vivo* agglutination or hemolysis — for lysis frequently goes hand in hand with agglutination provided alexin be present — resulting from the injection of erythrocytes of an incompatible donor. The donor's serum, on the contrary, although it may contain agglutinins for

* For sketches of the history of blood transfusion and appropriate bibliography see Wiener (18) and Snyder (34).

the recipient's corpuscles, usually produces no untoward effect, since the dilution which it undergoes in the total blood volume of the recipient is usually sufficient to render these agglutinins inactive. Hence, as Ottenberg (35) pointed out, it is possible to transfuse group O blood into persons belonging to any group, while members of group AB, since their serum contains no agglutinin, can receive blood of any group. Accordingly, individuals of group O are referred to as universal donors and those of group AB as universal recipients. Occasionally reactions have followed the transfusion of O bloods due to unusually high agglutinin titers which were still active even after dilution in the blood of the recipient. Therefore it is advisable to avoid the use of universal donors except in cases of emergency when no person of the same group as the recipient can be quickly found. When a universal donor is employed, an effort should be made to select one whose agglutinin titer for the recipient's cells is low.

The Blood Groups as Hereditary Factors

That the four classical blood groups are inherited according to Mendelian principles was first suggested by Epstein and Ottenberg (36) in 1908. Their data derived from the determination of the groups in parents and children of only two families were of course insufficient to establish the validity of their hypothesis. But the extensive studies of von Dungern and Hirschfeld (37), carried out two years later on seventy-two families, clearly demonstrated the fact that the groups were inherited according to the formula of Mendel. In the inheritance of these characteristics, the agglutinogens A and B appeared to be dominant to the agglutinins α and B.

The facts of blood group inheritance as they are recognized at the present time will be perhaps more easily comprehended if we briefly review the fundamental principles of genetics.*

All experimental evidence to date points towards the chromosomes as the bearers of the hereditary factors, or "genes." These bodies of chromatin material normally have a definite number, size, and shape for a particular species of the plants and animals. Man, for example, has 48 chromosomes, or 24 pairs. Under normal conditions, the chromosomes are paired, and the members of each pair are homologous in size and in shape, with the exception of certain cases in which there is a difference associated with sex. Further, there is much evidence for the conclusion that each gene occupies a definite location upon its particular chro-

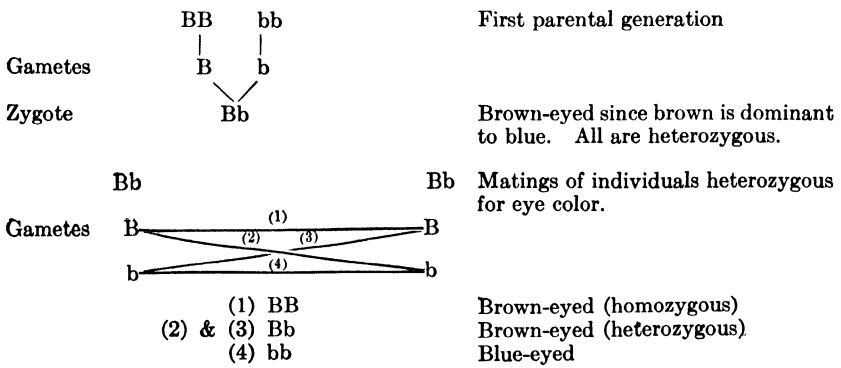
* This summary of the principles of genetics and illustrations of their application to the theories which have been offered in explanation of the hereditary mechanism of the blood groups has been prepared for us by Dr. M. R. Irwin, formerly of the Bussey Institute of Harvard University and now of the Rockefeller Institute. To him we are greatly indebted for this kindness.

mosome; genes on identical loci of homologous chromosomes are called allelomorphs.

At a certain stage in the development of the germ cells, a reduction in the number of the chromosomes takes place, with the result that the functional germ cells contain but half the normal number; *i.e.*, one member of each pair. This division of the pairs of chromosomes, which naturally includes the separation of the homologous genes, followed by the restoration of the full complement of chromosomes at the union of the sperm and the egg, offers a physical basis for the segregation and recombination of the hereditary factors — the basis of Mendel's Law. The operation of chance at reduction division for both male and female cells, and again at fertilization of the egg accounts for the ratios of the hereditary characters which appear in the progeny of hybrid parents.

Certain genes have the ability to obliterate the effect of their allelomorphs, when the two are present in the same individual. Thus, to employ an illustration for explanation only, if we agree that brown and blue eyes are the result of an allelomorphic pair of genes, it will follow that the children resulting from marriages of brown-eyed parents with only brown-eyed ancestry to blue-eyed mates will all be brown-eyed. The brown-eyed character is dominant to the recessive blue-eye. A dominant character may conceal the presence of a recessive factor, but the recessive character cannot conceal a dominant factor. Offspring of marriages between blue-eyed people will all have blue eyes.

A simple diagram will illustrate, representing the factor for brown-eyes by B, and for blue-eyes by b. A parent homozygous for brown-eyes has the factorial composition BB, for blue-eyes bb.



With this introduction into the most simple case of genetic ratios, let us use for further illustrations the factors determining the blood groups in human beings. We shall consider these under the different theories advanced for the explanation of the manner of their inheritance.

a. Independent inheritance. In this type of inheritance the various pairs of factors are located on different pairs of chromosomes. Let us assume that the factor A is responsible for the agglutinogen A, and a determines the agglutinin α. B determines agglutinogen B, and b agglutinin β. A and B when present in the same individual produce type AB, when both are absent (aabb) the type is O. A and B are dominant to their allelomorphs a and b, respectively. Further, let us take as a starting point the matings as given below:

	AABB	aabb	Parents
Gametes	AB	ab	
Zygote	AaBb		Type AB, but heterozygous for both characters
	AaBb	AaBb	Matings of such hybrids
Gametes	AB	AB	Possible combinations are each gamete with every other gamete (of mate) with equal chance of union
	Ab	Ab	
	aB	aB	
	ab	ab	

Without going into the details of the outcome of the different combinations possible between the gametes of dihybrid parents, it readily will be observed that the resulting progeny will have a varied factorial composition. Only four of the 16 possible combinations will produce homozygous types, AABB, AAbb, aaBB, and aabb, respectively. The others will be heterozygous for one or both characters.

b. Multiple allelomorphs. Referring to the previous definition of an allelomorph, we extend our concept to include more than a single pair of genes, a series, which act from identical loci on alternate characters. Only two of these genes may be present in any individual.

In the theory advanced to explain the inheritance of the blood groups on the basis of triple allelomorphs, factors A, B, and R are represented as the determiners of types A, B, and O, respectively; A and B together produce type AB, RR produces O. A and B are each dominant to R. It follows that an individual in so far as his blood group is concerned, may have any one of the following factorial complexes, with the possibility of producing germ cells as given:

	Germ Cells		
AA	A		
AB	A	B	
AR	A		R
BB		B	
BR		B	R
RR			R

c. Linkage. The term linkage is applied to those cases in which there is a measurable tendency for certain characters to occur together. The

best explanation of this phenomenon is that the factors for these characters are located on the same chromosome. As these characters, which exhibit linkage rarely, if ever, always occur together, evidence points to an interchange of parts of the homologous pairs of chromosomes. The strength of the linkage is determined by the relative distance between the loci of the respective factors; *i.e.*, the closer together, the stronger the linkage.

Let us use as factor symbols the same as assumed under independent inheritance, with linkage between A-B and a-b. The usual method of representing linkage between two factors to distinguish the type from independent inheritance is to write the genotype of the individual as AB.ab, denoting linkage between the factors as given. Without complete linkage, other combinations would be possible, as Ab.aB, Ab.ab, etc.

If we should follow through hypothetical matings of homozygous parents, we have below:

	AB.AB	ab.ab	Parents
Gametes	AB	ab	
Zygote	AB.ab		Type AB, but heterozygous
	AB.ab	AB.ab	Matings of such hybrids
Gametes Parental	AB	AB	
	ab	ab	
Non-parental (crossover)	Ab	Ab	
	aB	aB	

The combinations as they occurred in the original parents will be in excess over the non-parental types of blood groups. There is no known *a priori* ratio of the parental to the non-parental gametes; this can be determined only by actual count of the progeny from hybrid parents.

Von Dungern and Hirschfeld believed that the factors determining the four blood groups were inherited as two pairs of independent factors, A,α and B,β with the factor for the agglutinogen dominant to the agglutinin in each case. Their views were generally accepted until Bernstein (38, 39) in 1924 showed by means of a statistical analysis of the then very large body of available data that the frequency of group AB in a given population was significantly less than was to be expected on the basis of the theory of von Dungern and Hirschfeld. On the assumption that the factors determining the blood groups were not transmitted by independent pairs of allelomorphic genes, but rather as triple allelomorphs (see paragraph *b* above), Bernstein found that the theoretical frequencies agreed well with those actually found for group AB. Bern-

stein's conception of the genetic formulae of the groups are depicted in the following table:

Group	Genetic Formula
O	RR
A	AA AR
B	BB BR
AB	AB

It also became evident that Bernstein's theory could be subjected to experimental analysis by a critical study of the blood groups of children born from matings in which one of the parents belongs to group AB. On the basis of the hypothesis invoking two independent pairs of allelomorphs these matings should give children belonging to all of the four blood groups, as will be evident from the following table which gives the possible genotypes which could arise from the matings of hybrids containing both pairs of allelomorphs (see paragraph *a* above).

Groups

O	A	B	AB
aabb	AAbb Aabb	aaBB aaBb	AABB AaBB AABb AaBb

The theory of triple allelomorphs, however, precludes the birth of group O children from such crosses, since A and B are assumed to be dominant to the R allelomorph which determines group O. With this in mind a number of investigators have determined the blood group of the children of AB mothers, since this procedure eliminates the necessity for considering the paternal factor, which may be justly in doubt. As a result of fourteen such studies (18) which include 898 children of 627 mothers belonging to group AB, only three were found to belong to group O. The investigations in which these three cases were encountered were accomplished without a knowledge of the Bernstein theory, and it is possible that with greater care in respect to technique and repetition of the tests, these few exceptions might have proved invalid.

Since Bernstein's conception agrees better with observation and statistical analysis, it is now generally accepted by nearly all workers in this field. A third alternative has, however, been proposed by Furuhata and his associates (40), which may properly be regarded as a modification of Bernstein's theory. It differs from his in respect to the manner in which the agglutinins are inherited. According to the latter,

α and β agglutinin develop in every individual, irrespective of group. It is only the agglutinogens or their absence which are transmitted according to Mendelian laws. Since it is known that the agglutinogen appears first, whereas in many cases the agglutinin does not develop until some weeks or months following birth, Bernstein assumes that the agglutinin as it is formed reacts with the agglutinogen already present and is thereby absorbed, and thus eliminated, leaving the remaining antibody in the plasma. Since group specific antigen occurs not only in the red cells but in most of the other tissues of the body, it is not difficult to conceive of the effectiveness of this mechanism. Furuhata, however, believes like von Dungern and Hirschfeld, that the blood groups are inherited by two pairs of allelomorphic genes, with those determining the agglutinogens A and B dominant over those determining the corresponding agglutinins. But these are transmitted by three completely linked pairs: (Ab), (aB), and (ab) (see paragraph *c*, above), which, it is evident, correspond to Bernstein's three factors. We have as yet no conclusive evidence to indicate which theory is correct, but Bernstein's is perhaps to be preferred since it is more simple and is also more general, inasmuch as it permits an explanation for the occurrence of the anomalous agglutinins in the sub-groups of A as Friedenreich (41) has pointed out.

The agglutinogens M and N according to Landsteiner and Levine (42), who made the first studies on their inheritance, and other investigators, are inherited as simple Mendelian dominants conditioned in all probability by one pair of allelomorphs, M and N. These factors are inherited, therefore, exactly like A and B, except that an individual always possesses either one or the other, or both. There is no group analogous to group O. In well over 20,000 bloods none has been found possessing neither M nor N (43). The genetic formulae, therefore, are MM, NN, MN.

On the basis of what has been learned concerning the heredity of the blood groups and the new factors M and N, it is possible to draw four general conclusions:

1. The agglutinogens A and B cannot appear in the blood of a child unless they were present in the blood of one or both parents (von Dungern and Hirschfeld).

2. It is impossible for a member of group AB to be the parent of a group O child. Conversely, a group O parent cannot give rise to an AB child (Bernstein).

3. The agglutinogens M and N cannot appear in the blood of a child unless they were present in the blood of one or both parents (Landsteiner and Levine).

4. An M parent cannot give rise to an N child and, similarly, an N individual cannot be the parent of an M child (Landsteiner and Levine).

Blood Groups in Relation to Legal Medicine. By means of the genetic formulae and these general rules, based upon experimental evidence, it is possible in a certain proportion of putative matings to establish the fact that a child could not be the offspring of a certain individual. The converse, however, is not true. In no instance can it be shown that an individual is the parent of a given child. But it is apparent that negative evidence of this sort is of great value in legal controversies involving questions of paternity, or in cases where the identity of a child is in doubt, such as have occurred as a result of interchange in the infant wards of hospitals or intentionally by wet nurses.

In the table which follows and which we take from Hooker and Boyd (43), there is set forth the various blood groups which may arise in the offspring of each of the possible sixteen types of parent matings. Included also are the groups which could not occur as a result of such combinations of parents. Furthermore it will be observed that it is impossible for mothers belonging to group O to give birth to children of group AB and conversely mothers of group AB cannot have children

BLOOD GROUPS OF OFFSPRING POSSIBLE OR IMPOSSIBLE FROM ANY MATING COMBINATION

(After Hooker and Boyd)

	ALLEGED FATHER	KNOWN MOTHER	POSSIBLE CHILDREN FROM THEIR MATING	CHILDREN NOT POSSIBLE FROM THEIR MATING. DECISIVE FOR NON-PATERNITY	IMPOSSIBLE FROM THIS MOTHER IN ANY MATING
1	O	O	O	A, B, (AB)	AB
2	O	A	O, A	B, AB	
3	O	B	O, B	A, AB	
4	O	AB	A, B	(O), AB	O
5	A	O	O, A	B, (AB)	AB
6	A	A	O, A	B, AB	
7	A	B	O, A, B, AB		
8	A	AB	A, B, AB	(O)	O
9	B	O	O, B	A, (AB)	AB
10	B	A	O, A, B, AB		
11	B	B	O, B	A, AB	
12	B	AB	B, A, AB	(O)	O
13	AB	O	A, B	O, (AB)	AB
14	AB	A	A, B, AB	O	
15	AB	B	A, B, AB	O	
16	AB	AB	A, B, AB	(O)	O

The letters designate the blood-types of the respective individuals. Those in parentheses, in column 5, could not be children of the corresponding mothers (column 3) in any mating.

belonging to group O, irrespective of the group to which the father belongs. In the cases of A by B matings children of each of the four groups are possible, and therefore non-paternity cannot be established without recourse to the determination of the M-N agglutinogens, which may occasionally show that the alleged father could not be the true parent of the child.

Based on the known frequencies of the four blood groups among a given population, calculations of the chances of proving non-paternity in the case of men belonging to each of the groups have been carried out.* Hooker and Boyd have published the following table of probabilities which indicate that on the average in a population such as that found in the United States or Western Europe the chances are 6 to 1 against a man of unknown blood group disproving paternity. His chances are much greater if he happens to belong to group AB and very small if he is of group A.

PROBABILITIES OF PROVING NON-PATERNITY WHEN BLOOD GROUP OF WRONGFULLY ACCUSED MAN IS KNOWN

(After Hooker and Boyd)

GROUP	APPROXIMATE PER CENT IN THE UNITED STATES	PROBABILITIES
O	45	$\frac{1}{5}$[1]
A	42	$\frac{1}{17}$
B	10	$\frac{1}{7}$
AB	3	$\frac{1}{2}$
(If unknown		$\frac{1}{7}$)

[1] $\frac{1}{5}$ = 1 in 5 = 20 per cent.

The average chance of exonerating a man by means of blood tests was considerably increased by the discovery of the M and N agglutinogens and the analysis of the conditions governing their inheritance. In the table which we have modified after Wiener (18), the various agglutinogens which can or cannot appear in the red cells of the children resulting from the possible matings are summarized.

AGGLUTINOGENS OF PARENTS	AGGLUTINOGENS WHICH OCCUR IN THE CHILDREN	AGGLUTINOGENS WHICH MAY NOT OCCUR IN THE CHILDREN
MN × MN	MN, M, N	—
MN × N	MN, N	M
MN × M	MN, M	N
M × N	MN	M, N
M × M	M	MN, N
N × N	N	MN, M

* Literature: Wiener (44), Koller (45), Hooker and Boyd (43).

According to Wiener, who has calculated the chances of proving non-paternity by means of the presence or absence of these factors in mother, child, and putative father, a man whose M or N agglutinogen are unknown has about one chance in five of demonstrating non-paternity. If he belongs to type MN, he has no chance at all, whereas if he is M, his chances are one in three, and if N, two in five. Wiener has also determined the probabilities when both the classical blood groups and the M and N factors are considered together. It will be seen from the table that in most cases the chances of establishing non-paternity are significantly increased. This is clearly illustrated by comparing the average probability in the case of the blood groups alone, the M and N factors alone and when they are combined.

Group and Type of Putative Father	Chances of Proving Non-paternity	
	Per Cent	Expressed as Chances (Approximate)
O M	50.	1 in 2
O N	54.6	3 in 5
O MN	23.5	1 in 4
A M	39.6	2 in 5
A N	45.1	3 in 7
A MN	7.7	1 in 12
B M	44.1	3 in 7
B N	49.3	1 in 2
B MN	14.6	1 in 7
AB M	60.7	3 in 5
AB N	64.3	2 in 3
AB MN	39.9	2 in 5
Unknown (average)	31.9	1 in 3

Considerable evidence has accumulated which indicates that the agglutinogens of the sub-groups of A are inherited according to Mendelian principles. Insufficient data make it impossible at present entirely to rely upon the theory of inheritance proposed by Thomsen, Friedenreich, and Worsaae (46) to account for their distribution among the offspring of one or both parents belonging to groups A, A_2, AB, A_2B. Should further studies establish its validity, which is highly probable, these additional factors will still further increase the chances of determining whether or not a man can be the father of an alleged child.

For some years, the evidence derived from blood grouping has been accepted widely in the law courts of many European countries. In the United States, on the other hand, its acceptance has been slow. Although there are now indications that blood grouping tests will find a much more extensive use in legal medicine, since it is clear that they

are remarkably accurate, provided the techniques are carried out as they always should be, by skilled and experienced workers.*

The application of these procedures in court cases is not confined to those involving questions of paternity, for by their use non-maternity may in some instances be affirmed, blood stains identified as belonging to a given group, or parentage of an infant may be indicated where this is disputed by two groups of alleged parents.

We have presented this somewhat detailed discussion of the medico-legal aspects of the subject not only because of its immediate applications but because it serves well to illuminate the underlying principles of the inheritance of the blood groups and the agglutinogens M and N.

The Distribution of the Blood Groups among Different Races. The analysis of a large number of human blood specimens from different racial groups has yielded data of considerable anthropological interest. The first of these studies were made by the Hirschfelds (47) during the war, on troops which represented racial groups of widely different origins. It was found that the frequencies of the four blood groups varied significantly in certain cases. Most striking was the difference in the frequencies of groups A and B in peoples from Europe and Asia, which becomes readily apparent from an examination of the following table. It will also be observed from the data which are included that pure line American Indians are characterized by a tremendous predominance of group O.

TABLE ADAPTED FROM HIRSCHFELD (48) AND OTTENBERG AND BERES (49)

	O	A	B	AB
English	46.4	43.4	7.2	3.1
French and Germans	40 — 43.2	42.6 — 43	11 — 12	3 — 5.
Russians	40.7	31.2	21.8	6.3
Arabs	43.6	32.4	19.	5.
Chinese [1]	30.	25.	34.	10.
American Indians [2]	77.7	20.2	2.1	0
Amer. Indians,[3] unmixed stock	91.3	7.7	1.	0
Australian aborigines [4]	51.	38.5	3.	1.5

[1] Lui and Wang. *Nat. Med. Jour. China*, vi, 1920, p. 118.
[2] Coca and Deibert. *Jour. Immunol.*, viii, 1923, p. 478.
[3] Snyder. *Am. Jour. Phys. Anthropol.*, ix, 1926, p. 233.
[4] Tebbutt and McConnell. Cited from Ottenberg and Beres.

Since these earliest researches, many investigators have carried out similar studies. Summaries of these may be found in the treatises of Snyder (34), Wiener (18), and Steffan (50). As a result, adequate

* For critical discussions of the reliability of blood grouping tests, the reader is referred to Wiener's monograph (18) and the paper of Hooker and Boyd (43).

samplings of practically every race in the world have been taken. On the basis of this material attempts have been made to trace the movements and intermingling of peoples of supposedly diverse origins as well as to divide the peoples of the world into broad racial types. We cannot here give a detailed treatment of this matter, and refer the reader to the literature just mentioned and to the older reviews of L. Hirschfeld (48) and Ottenberg and Beres (49). In a general way the grouping of peoples according to the relative frequencies of the genes A and B agrees fairly closely with that based on other anthropological criteria. A not inconsiderable number of exceptions occur, however, and two races with the same frequencies may be found which in other respects are obviously quite unrelated. For anthropological purposes blood grouping nevertheless has undoubtedly proved of value, especially as it is a character which is not subject to conscious selection in mating. But, as Snyder observes, it should not be unduly emphasized in contrast with others such as pigmentation, hair form, and cephalic index.

In considering the origin of the blood groups, Bernstein, largely on the basis of the predominance of group O in the American Indians, suggested that the original races of man were pure recessives belonging to this group, from which the other groups split off by mutation. Ottenberg, quite correctly, it seems to us, approaches this theory with conservatism, basing his skepticism particularly upon observations of Landsteiner and Miller (51), who have demonstrated the agglutinogens A and B in chimpanzees and orangs. Furthermore, Matson and Schrader (52) have found a very high incidence of A among supposedly pure-blooded members of the tribe of Blackfeet Indians.

Landsteiner and Levine (53) were the first to reveal differences in the proportion of the M and N factors among American Indians and certain other races such as Germans, Japanese, and Negroes. All of the latter have about 20 per cent of type N and 30 per cent of type M, whereas the respective frequencies among Indians are approximately 5 per cent and 60 per cent. Recently Boyd and Boyd (25) in a relatively small series (106) of Mohammedans in Assiut found a significantly low frequency of type N compared with the Copts living in the same locality. Considerably more information will be necessary before the true state of affairs in respect to the ratio of M to N in different races can be recognized.

Blood Differences in Lower Animals. Iso-agglutinins and agglutinogens characteristic of groups within the species or even apparently of individuals have been observed in the blood of lower animals. We have already mentioned the work of Ehrlich and Morgenroth (1), who demonstrated isolysins in goats. Klein (54), in 1902, found individual differences in the blood of horses, and his results have been more recently

substantiated by those of Hirschfeld and Przesmycki (55) and Schermer (56). In 1910 Boycott and Douglas (57) reported the existence of iso-agglutinins in the blood of rabbits, but Snyder (34) in a large series of experiments failed to detect their presence. Like rabbits, cats appear to have little or no normal agglutinins for the cells of other cats, although differences in the agglutinogens of the red cells probably exist since isolysins develop following repeated cat to cat transfusions (58). Rabbits, too, differ in respect to the antigen in the red cells as Levine and Landsteiner (59) have shown. It is also of interest that some rabbits have normal agglutinins which react specifically with the cells of the various human blood groups (60, 61). Dogs, according to von Dungern and Hirschfeld (62), although lacking or exhibiting only weak iso-agglutinins can be divided into four groups by means of appropriate immune sera. The blood of the guinea pigs (63) and mice (64) appears to be without either isoagglutinins or substances within the cells which are capable of distinguishing between groups or single animals. Among cattle iso-agglutinins may occur but these are usually weak (63). On the other hand, an amazingly large number of differences in the red cells of various individuals can be revealed by Ehrlich's method of iso-immunization. Todd and White (65) injected cattle with large quantities of the blood of other animals of the same species. A number of sera thus obtained were pooled. It was found that this pooled serum then caused lysis of the bloods of all cattle tested. If then the serum was absorbed with the red cells of a single individual, until all hemolysin for those cells was removed, the absorbed serum nevertheless lysed the erythrocytes of 110 other cattle. Todd and White took this to signify that each individual cow or steer possessed in his cells substances specific for itself. Wiener (18), however, has shown on theoretical grounds that this assumption is not inevitable, since the same results might be obtained if one postulated only a relatively few agglutinogens or lysinogens distributed in different ways in the cells of different individuals. Todd and White found that sheep behaved in a similar manner, and Landsteiner and Miller (66) and Todd (67, 68) demonstrated a very great number of individual differences between the bloods of chickens. The factors upon which these depend appear to be inherited.

In pigs three blood groups have been reported by Bialosukuia and Kaczkowsky (69). Weszeczky, however, although observing occasional isoagglutination, was unable to distinguish definite groups. Again, Fischer and Klinkhart (70) and Buchbinder (71) have by the iso-immunization technique found differences between the cells of some of the lower monkeys.

Great biological interest pertains to the discovery by von Dungern and Hirschfeld (72, 73) and Landsteiner and Miller (51, 74, 75, 76) that

the bloods of anthropoid apes contain agglutinogens and iso-agglutinins which serologically at least seem to be identical with those found in man. The latter authors found among fourteen chimpanzees three which belonged to group O and eleven corresponding to group A. Among six orangutans, two were identified as group A, two as group B, and two as group AB. One gibbon belonged to group A. Cross absorption tests with ape iso-agglutinins and human cells and human agglutinins and A cells indicated a complete identity between these entities. Later communications of Landsteiner (77) confirmed and extended these findings. This adds, as he suggests, another piece of evidence of the relationship of man and the higher apes and also promotes speculation on the possible evolutionary significance of the similarity of antigenic properties. So far all four groups have not been observed among the various species of anthropoids. Since relatively few animals have been studied, this does not necessarily imply that none possesses them.*

Factors corresponding to the M and N agglutinogens of human blood have been noted among the higher apes and certain of the lower monkeys. In their first communication on the properties of these elements Landsteiner and Levine (21) reported that both M and N occurred in the bloods of 10 chimpanzees which they studied. Landsteiner and Wiener (79) and Wiener (80) have recently confirmed these observations and also have demonstrated M but not N in the bloods of baboons, orangutans, gibbons, and certain species of lower monkeys such as *M. rhesus*, the green monkey, and the white-faced monkey. These factors in apes and monkeys are similar but not identical with those found in man. So far neither M nor N has been detected in the bloods of animals other than the primates.

The Presence of Group Specific Substances in Tissues Other Than the Erythrocytes

Beginning with the demonstration by Landsteiner and Levine (81) of substances in spermatozoa which could specifically inhibit the agglutinative action of A or B serum, it has been shown that the tissues of nearly all the organs of man as well as various secretions such as tears, urine, and milk contain these antigenic factors which may be extracted with aqueous and in many cases with alcoholic solutions (18). The usual technique employed in determining their presence depends upon the capacity of the substance to absorb the agglutinins from an A or B serum. For example, if an extract containing B specific substance

* An exhaustive discussion concerning blood differences in animals, with extensive bibliographical material, will be found in the review by Thomsen in Steffan's *Handbuch* (50). In this connection a paper by Landsteiner (78) may also be consulted which summarizes much of the literature up to the time of its publication.

is mixed with A serum, and B cells are subsequently added, no agglutination of the latter will follow. By this method various authors have detected group substances in nearly all the tissues which had been almost if not completely freed of blood by careful washing. The quantitative experiments of Schiff (82) reveal a pronounced difference in the amount of group specific substance present in different organs. He found that the pancreas contains by far the most. Only about 7 per cent of this amount is present in lung and heart, while the brain contains only about one fifth of the quantity found in these two organs. These data indicate quite clearly, we believe, that the group substance is without doubt present in the tissue cells and not in the small residuum of blood which might remain following careful washing.

The iso-agglutinins also are not confined to the blood serum but appear in other body fluids having a high protein content. They have been found in milk lymph, exudates, and transudates. In urine they do not occur unless pathologic conditions lead to the abundant elimination of albumin, nor have they been found in significant quantities in the spinal fluid, tears, saliva, or spermatic fluid.

From this standpoint little work has as yet been done on the M and N substances. According to the report of Zacho (83), they are not extracted by either salt solution or alcohol from normal tissues or benign tumors, but strangely enough, they appear in watery extracts of malignant growths.

The chemical nature of the group specific substances has been alluded to in Chapter II. From their general properties they are probably not protein in nature. There are many reasons for regarding them as carbohydrate haptenes, possibly associated or linked with lipoidal elements.

The facts concerning the blood groups are of general interest to the immunologist entirely apart from their immediate practical applications, since they afford an undisputed instance of the production of antibodies which is due solely to intrinsic physiological processes and not to the introduction of a foreign antigenic substance. This is of great significance in respect to the unsolved problem of whether or not the multitudinous natural antibodies of man and the animals against bacteria and various other substances always arise as a result of contact or in some cases as the products of internal processes analogous to those leading to the formation of the iso-agglutinins.

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CHAPTER XI

THE PHENOMENA OF PHAGOCYTOSIS AND INTRACELLULAR DESTRUCTION OF LIVING ORGANISMS

Definition. The phenomenon to which the term *phagocytosis* refers consists in the ingestion of a particle of foreign material by a living cell. In its strictest sense there is no implication in the meaning of the word concerning the fate of the ingested particle, which may or may not be digested and destroyed, depending upon its individual properties. We will include in this chapter, however, a discussion of this latter function of the phagocyte.

General Biological Significance. Phagocytic capacity is obviously an attribute of those unicellular organisms, such as amebae, which obtain a part of their food in the form of insoluble fragments. In addition many of the various types of cells comprising the complex structure of the metazoan body retain this function which in certain of the primitive forms, such as the coelenterates, is still principally directed toward the intake of nutritive substances. In the more highly developed species, however, this mode of ingestion of foodstuffs is replaced by extracellular enzymatic digestion and the direct absorption of the soluble products from the intestinal tract. In such organisms the activity of the phagocytic cells finds its chief application in the removal of a variety of particulate matter which, emanating either from an endogenous or exogenous source, consists of substances which are useless or actually harmful to the normal physiological processes of the body.

From our standpoint it is, of course, the phagocytosis of the latter class of harmful substances, as represented mainly by the parasitic agents of disease, which will be our primary interest in this and the following chapter. Since the classical researches of Metchnikoff (1, 2), who clearly demonstrated the ability of the phagocytic cells not only to ingest but also to destroy many pathogenic organisms, their activity in this respect has been regarded by the majority of immunologists as one of the most fundamental components in the complex processes which represent the "resistance" of the host to infection.

It must not, however, be forgotten that just as the various serological phenomena which have been described have an extensive biological

significance apart from their importance in relation to bacterial invasion, so the process of phagocytosis should also be looked upon as an attribute of the animal and vegetable cell possessing essential physiological functions entirely apart from the disposal of an invading parasite. Metchnikoff himself, a zoologist by training, was the first to make clear and to emphasize these broad conceptions of the place of phagocytosis among general biological phenomena.

Thus during normal life in the higher animals, there is constant fragmentation of red blood cells. The resulting debris is taken up largely by the phagocytic cells of the spleen, but also to a less degree by those of the liver, bone marrow, and lymph nodes (3, 4.) Mononuclear cells of the tissues and blood may also take part in the removal of degenerative erythrocytes (5). The rapid return of the puerperal uterus to the normal state appears due at least in part, as Helme (6) showed, to phagocytosis of the hyperplastic uterine musculature during this period. Similarly, according to Matschinsky (7), the atrophy of ova in the ovary is followed by active phagocytosis of portions of this structure. Metchnikoff (8) asserted that the whitening of the hair in old age was due to the phagocytosis of the pigment. There is a considerable body of experimental evidence (9) which strongly suggests that the formation of bile pigment from hemaglobin is accomplished by certain of the phagocytic cells of the so-called reticulo-endothelial system. Reference has already been made (see page 162) to this system of cells, characterized most distinctively by their phagocytic properties, as a possible anatomical site for the formation of antibodies.

In lower animals the operation of phagocytosis in normal physiological changes is well illustrated. In some insects (diptera) the destruction of larval organs, useless to the adult, is effected by the action of phagocytic cells, while a similar process accompanies the transformation of the tadpole to the adult in amphibia (10).

In many pathological conditions other than their direct action on the invading organism the action of the phagocytes is frequently indispensable in the repair of tissue damage. For example, after the extravasation of blood into the tissues the leucocytes rapidly enter the area and an engulfment of blood corpuscles occurs, followed by a process of digestion analogous to the digestion of such cells by intestinal hemamebae. That the leucocytes are concerned in the destruction and resorption of dead tissues was particularly emphasized by Leber (11). The phagocytes may therefore be regarded as cells retaining primitive characteristics for definite physiological purposes.

Phagocytes and Resistance to Infection — Fundamental Observations of Metchnikoff. Early investigations into the fate of bacteria within the infected animal body were largely carried out by pathological anatomists, and the observation of the presence of microorganisms within the cells of the animal and human tissues was definitely made as early as 1870. Hayem (12), Klebs (13), Waldeyer (14), and others saw leucocytes containing bacteria but failed to interpret this in the sense of possible protection. The process was regarded rather as a means of transpor-

tation of the bacteria through the infected body, or it was assumed that possibly the microorganisms had entered these cells because of the favorable nutritive environment thus furnished.

The first to suggest that such cell ingestion might represent a method of defense was Panum (15), who referred to it as a vague possibility. A similar but more convincing expression of this opinion was made in 1881, according to Metchnikoff (2), by Roser, although no experimental support for his contention was presented.

The significance of cell ingestion as a mode of protection against bacterial invasion, therefore, was hardly more than a vague suggestion when Metchnikoff began to experiment upon the cell reaction which followed the introduction of foreign material, living or dead, into the larvae of certain starfishes (Bipinnaria). Pathologists at this time held complicated views of inflammation which involved complex co-ordinated reactions of vascular and nervous systems, and Metchnikoff's primary purpose was to observe reactions to irritation in simple forms devoid of specialized vascular or nervous apparatus. He (1) noted in such transparent simple forms of life that the foreign particles were rapidly surrounded by masses of ameboid cells and reached a conclusion which, in his own words, is expressed as follows:

"L'exsudat inflammatoire doit être considéré comme une réaction contre toutes sortes de lésions et l'exsudation est un phénomène plus primitif et plus ancien que le rôle du système nerveux et des vaisseaux dans l'inflammation."

He compared the process of cell ingestion or phagocytosis of foreign particles to that taking place in the most simple intracellular digestion which occurs in unicellular forms, a hereditary cell function now specialized in certain mesodermal cells, and passed on in the evolution of higher forms to other specialized cells. And indeed in higher animals the leucocytes which carry on this phagocytic process may be considered as representing a primitive form of cell, since they are only nucleated elements of the body which wander from place to place, and are independent of nervous control. In 1883, at the Naturalists' Congress in Odessa, Metchnikoff (16) first expressed his views and communicated the first of the researches upon which our modern conception of phagocytosis is based.

His earlier studies were carried out with a small crustacean, the daphnia, in which he studied the reaction which followed the introduction of yeast cells. He observed the struggle which ensued between the ameboid leucocytes of the crustacean and the infecting agents and determined that complete enclosure of the yeast within the leucocytes assured protection to the daphnia, while a failure of this process, either from fortuitous causes or because of too large a quantity, or too high a virulence of the infecting agents, resulted in disease and death. This early work

forms the beginning of a train of investigations to which we owe most of the basic facts we possess concerning the role of the phagocytic cells in the protection of the body against infection.

The Types of Phagocytic Cells — Metchnikoff's Classification. Many studies have been made to determine which cells of the body of higher animals can take in and digest foreign particles and to classify them according to this power. Metchnikoff has distinguished between the "motile" and "fixed" phagocytes, the former the leucocytes of the circulating blood, the latter certain connective tissue cells, endothelial cells, splenic pulp cells, and certain cellular elements of the lymph nodes, the neuroglia tissue, and, in fact, all phagocytic cells which are ordinarily confined to some definite localization in the body. Among phagocytic cells Metchnikoff further distinguished between "microphages," by which he designated the polymorphonuclear leucocytes of the circulating blood and "macrophages." The macrophages include the fixed cells mentioned above, together with the large mononuclear elements of the blood, and the wandering tissue cells, the clasmatocytes of Ranvier, in short, all phagocytic cells except the microphages.

This original division of the phagocytic cells by Metchnikoff into two main groups on the basis of nuclear morphology has proved entirely valid. On the other hand, the criterion of a difference of phagocytic activity toward various classes of substances also invoked by him as a basis for a classification into these groups, has not been entirely upheld by subsequent researches. As will be pointed out below, Metchnikoff's conception that at least in acute bacterial infections the rôle of the microphage consisted in taking up the bacteria while that of the macrophage involved almost exclusively the phagocytosis of dead tissue and moribund cells, has been shown to be contrary to the observed behavior of these types of cells both *in vivo* and *in vitro*.

The combined studies of cytologists and immunologists have revealed the origin and development of the microcytes from well-defined parent cells (myeloblasts) in the bone marrow and the important activity of the neutrophile in the phagocytosis of bacteria. Among earlier observers (17) some have described the eosinophile as exhibiting distinct phagocytic properties, while others have denied this. More recently Jacobsthal (18) and Strumia and Boerner (19) have noted definite phagocytosis of various bacteria by eosinophiles. Little is known concerning phagocytosis by basophiles. But Ringoen (20) and Strumia (19) have observed microorganisms within basophiles.

Not so distinct are the various subdivisions of the macrophage group. With the exception of the nervous tissue cells, and possibly the dust cells and septal cells of the lungs, it is generally agreed that the cells of mesenchymal origin arise from an undifferentiated parent cell or cells,

but the genetic relationships between the various morphologically differentiable components of the class even today are disputed by various authors. This group of cells since Metchnikoff's early work and Ranvier's description of the so-called clasmatocytes or wandering tissue cells, has been the object of increasing interest and investigation. Principally because of their marked phagocytic activity, but also because of the apparent association of many with the disposal of worn-out red blood corpuscles and leucocytes, and a certain amount of experimental evidence pointing to them as the site of antibody formation, Metchnikoff and his co-workers were inclined to regard these various cells as forming a system with similar physiological properties. Further evidence for considering them as components of an integrated system was afforded by the experiments of Ribbert (21), Goldmann (22), and others on their peculiar property to take up more or less large quantities of vital dyes, such as lithium carmine, after injection into the living animal (intravital staining). On the basis of these investigations Aschoff and Landau in 1913 grouped the cells of Metchnikoff's macrocytes together with certain other cells which were vitally stained but to a lesser degree, under the general heading of the "Reticulo-endothelial metabolic apparatus." Although the histological relationships between the cells of this system, and indeed the question as to whether they represent a true physiological unit, are far too complicated and unsettled to be here summarized satisfactorily, Aschoff's (23) description and classification of the various cell types on the basis of dye storage is of value in describing more precisely the elements of Metchnikoff's macrophage system, and in considering the kinds of cells, the great importance of which in the removal of bacteria from the body fluids and tissues has only recently been fully realized. According to Aschoff the following group of cells of mesenchymal origin comprise the reticulo-endothelial system:

1. The reticular cells of the splenic pulp, the cortical nodules and pulp cords of the lymph nodes, and ultimately the remainder of the lymphatic tissue. (These cells do not store vital dyes as easily or as quickly as the following groups.)
2. The endothelial cells of the sinuses of the lymph nodes, of the blood sinuses of the spleen, of the liver sinusoids, and of the capillaries of the bone marrow, adrenals, and hypophysis. This group is the most active of all in the storage of dye.
3. The histiocytes, *i.e.*, the motile inhabitants of the connective tissue, the clasmatocytes of Ranvier. These cells store dye almost as readily as those of group 2, particularly if they be in a special state of activity.
4. The splenocytes or splenic pulp cells and the dye-storing monocytes (endothelial leucocytes, blood histiocytes).

Aschoff characterized groups 1 and 2 as the reticulo-endothelial system, in the narrow sense of the term, on the basis of similarity of function,

since these cells form the reticulum and line blood and lymph sinuses. Because there is considerable evidence pointing to a close relationship between these cells and those included in groups 3 and 4, Aschoff included the latter in the reticulo-endothelial system in the broader sense. The relationship between the monocytes of the blood (group 4) and the histiocytes of the tissues (group 3) has, since the differentiation of these cells on the basis of supravital staining by Sabin and her co-workers (24, 25, 26), been the cause of controversy and of the establishment of two opposing schools. Of these the one represented by Lewis (27) and others regards the histiocyte, the monocyte, and the epithelioid cells of tubercles as merely different phases of the same cell type. Sabin and those who share her views, on the other hand, have until recently held that they are very distinct types of cells in respect to derivation, cytological characteristics, and function. In 1932, however, Sabin (28) stated that although she considers them to be different types, largely on the basis of the disparity in the manner in which they digest foreign materials, nevertheless they appear to be derived from the same primitive stem-cell (connective tissue cell) and that they are functionally very closely allied. She moreover concludes that the monocyte may give rise to the histiocyte. Forkner (29) asserts that the monocyte is not stained by vital dyes, and therefore doubts the propriety of including it in the reticulo-endothelial system. Since, admittedly, both cells readily ingest a large number of substances, the question of relationship would not be, perhaps, of great interest to the immunologist had not Sabin (30) asserted that whereas the monocyte took up the tubercle bacillus, it could not destroy it, and indeed formed a nidus for its future proliferation, while the histiocyte both phagocytes and digests this organism.*

The fibrocytes of the connective tissue and the endothelial cells of the blood and lymph vessels, although mesenchymal in origin, were excluded by Aschoff from the reticulo-endothelial system on the basis of their very limited ability to store dyes and differences in functional behavior. More recent work suggests, however, that the fibrocytes of the loose connective tissue may be transformed into histiocytes (31, 32, 33), and that in tissue culture these cells may become strongly phagocytic (32, 34). It is therefore possible that the fibrocytes should be assigned to the reticulo-endothelial system. Although Aschoff does not include the common endothelial cells of the blood and lymph vessels in the system, Mallory (35, 36, 37) and his followers assert that mononuclear phagoctyes may develop from these sources ("endothelial leucocytes"). Others (38, 39) have denied this possibility as well as any phagocytic activity

* For further references to the literature on the monophage-macrophage question see Forkner (29).

on the part of the vascular endothelium itself, although it is admitted that following intravenous injection of india ink, carbon particles may collect in these cells (40, 41, 42, 43). This phenomenon, however, is not regarded by the majority of authors as true phagocytosis but a kind of passive penetration of the cell by the particles. Hammerschmidt (44), however, working with a new species of the corynebacteria called by him *corynebacterium mûrisepticum*, which proved highly virulent for mice, found that the capillary endothelium took up these organisms in large numbers, while the cells of the reticulo-endothelial system, with the exception of the Kuppfer cells, remained inactive. In view of the large quantity of experimental data, part of which will be subsequently summarized, that show without doubt that the R–E system is of primary importance in the phagocytosis of bacteria within the circulating blood, Hammerschmidt's results must be regarded with great caution. Considering the available evidence, we cannot at present attribute to the cells of the general vascular and lymphatic endothelium any major participation in this process. Although believed by many writers to be derived from the epithelium, the septal cells of the pulmonary alveoli, which are definitely phagocytic, are regarded by others as mesenchymal elements (45). Therefore there is some basis for including them in the reticulo-endothelial system as Gay (46), for example, has done.

Many other types of cells have been observed to act as phagocytes, or at least to contain various sorts of foreign materials with which they have been brought in contact. Thus Erdmann (47) makes the broad statement that all mesenchymal cells may, under certain conditions, act as phagocytes. And Aschoff (23) affirms that occasionally every possible cell will "swallow" coarse foreign elements. Bratiana and Guerriero (48) have observed the phagocytosis of carmine and melanin particles by the epithelial cells of the acini following injection of these substances into the lactic ducts of the mammary gland. A phagocytic function has been ascribed by some to megakaryocytes and denied by others (49, 50), while Sugiyama and Tatibana (51) have noted a similar property of the epithelial cells of the kidneys. A review of the literature concerning the phagocytic properties of the glia cells, together with experimental observations of their own, is given by Cramer and Alpers (52), who assert that the oligodendroglia as well as the microglia are concerned in the ingestion of fat following secondary degeneration of the spinal cord. It is most probable that this ingestion by epithelial cells, like the endothelial cells of capillaries and lymph vessels, represents a purely passive type of phagocytosis in which the particle gradually becomes embedded in the protoplasm, and in which active participation of the cell which distinguishes the members of the microphage and reticulo-endothelial or macrophage systems is entirely lacking.

From the foregoing sketch it is clear that a large variety of cells distributed widely throughout the body may, under certain conditions, appear to be phagocytic. Among them, those which are known to be chiefly involved in the defensive reactions of the organism to infectious agents are included in the following table, which will serve as a summary, without suggesting genetic relationship between individual members of the group.

PHAGOCYTIC CELLS OF THE BODY DISTINGUISHED BY THEIR MARKED ABILITY TO INGEST PARTICULATE MATTER (ORIGINATE FROM THE EMBRYONIC MESENCHYMA)

MICROPHAGES (Granulocytic elements of the circulating blood)			MACROPHAGES (Mononuclear cells: fixed and wandering cells of the tissues and of the blood)				
Neutrophiles (most active)	Eosinophiles (conflicting reports as to activity)	Basophiles (slightly active)	Fixed endothelial cells of the blood and lymph sinuses of certain organs (most active)	Mobile histiocytes of the connective tissue (very active)	Septal cells of the lung (probably of mesenchymal origin — very active under certain conditions)	Splenic pulp cells and blood monocytes (moderately active)	Reticular cells of spleen and lymph nodes (least active)

Techniques for Studying Phagocytosis. It is a relatively simple matter to demonstrate the occurrence of the phenomenon of phagocytosis either *in vitro* or in the animal body. On the other hand, it has proved much more difficult to secure accurate quantitative information concerning the numbers of particles ingested under conditions in which all factors which might influence the reaction are satisfactorily controlled, thus enabling the observer to vary only one known element in a complex system and to observe the effect of such variation. The most precise data obviously can be gained *in vitro*, where measured amounts of bacterial suspensions can be added to known quantities of phagocytes and any material the effect of which in promoting or inhibiting phagocytosis it is desired to study. In their fundamental studies Denys (53, 54) and his colleagues, who first demonstrated the phagocytic promoting property, or, as Neufeld later termed it, the bacteriotropic action of specific antisera, observed the action of leucocytes of the shed blood on the corresponding organism in the presence of anti-streptococcic serum. Leishman (55) in 1902, apparently without any knowledge of the work of Denys, devised a similar method by which he was enabled to note the phagocytosis of staphylococci by the leucocytes of human blood. It consisted in taking small quantities of the blood of patients and mixing these in capillary pipettes with equal volumes of bacteria suspended in salt solution and incubating them together on a glass slide under a cover slip ringed with vaseline. At the end of incubation the

preparations were smeared upon slides and stained by Leishman's modification of the Romanowski method, the number of bacteria in a large series of leucocytes counted and an average taken. This method had many serious flaws, chief among them being the tendency to coagulation of the preparations and the fact that, in each test, the fluid constituents as well as phagocytes, both of them variable factors, came from the same individual. While, therefore, it was possible to estimate an increase or decrease of general phagocytic power, it was impossible to analyze this in reference to its dependence either upon the condition of the cells, or that of the plasma or serum. Moreover, the relation of the number of leucocytes to that of bacteria in individual tests necessarily differed, and this added a variable factor which rendered it impossible to compare any two experiments. In spite of these difficulties, however, Leishman succeeded in establishing in staphylococcus infection, that increased resistance was accompanied by increased energy of phagocytosis. But Leishman interpreted his results on Metchnikoff's erroneous "stimulin" theory that an increase in phagocytosis is due to an enhanced activity of the leucocytes rather than to a serum constituent.

The subsequent studies of Wright, which began at the point at which Leishman stopped, had, as their main result, the discovery of the opsonins, *i.e.*, constituents of normal serum which promote phagocytosis, and the confirmation of Denys' conception of the mechanism of co-operation between serum and leucocytes in phagocytosis. In order to carry out these studies the technique of Leishman was inadequate, and Wright's first task was to modify it in such a way that reasonably accurate comparative estimates of phagocytosis could be made.*

Briefly, Wright's early technique, which he applied for many years in the estimation of the results of vaccine treatment in a number of clinical conditions, consists in obtaining serum from the patient and sera from a number of supposedly normal individuals, setting up in capillary tubes equal quantities of (1) suspension of washed leucocytes from one normal individual, (2) the various sera to be tested, and (3) bacteria emulsion. After incubation the phagocytic mixtures containing normal sera are pooled, smears are made from this pooled mixture, and that containing the patient's serum stained, and in each case the bacteria engulfed by an arbitrary number (usually 50 or 100) of polymorphonuclear leucocytes are counted. The average number of bacteria per leucocyte is then calculated, and is taken as the *phagocytic index*. The ratio of the phagocytic index of the patient's serum to the phagocytic index of the normal pooled mixtures is the *opsonic index*. Various modifications and adaptations of the method have been published subsequently by Wright.

* The details of Wright's techniques and their subsequent modifications are given by A. E. Wright and L. Colebrook (56), and by A. Fleming (57).

It is obvious that by the use of such techniques, although they represent definite improvements compared with those of Denys and Leishmann, serious inaccuracies must frequently result. Phagocytosis can only take place after contact between cell and particle has occurred. Such contact may be brought about either by agitation of the mixture, in which case it is the result of a purely physical action, or under motionless conditions, by collision which may depend upon chance meetings as the leucocyte crawls about on the surface of the glass, or by definite chemotactic factors, which will subsequently be described, that lead to the direct advance of cell toward particle. With such methods as Wright's, in which no uniform and constant mixing is involved, the chance of contact between cells and particles may vary considerably, making it difficult to compare the results.

In addition to the large errors which may be induced by improper mixing, Wright's procedure fails to yield conclusive evidence concerning the fate of an ingested particle, which if it be a bacterium or other living organism, is of foremost interest to the student of immunological processes. A further difficulty inherent in the method lies in standardizing the "normal" serum control, since various sera from normal individuals vary widely in their capacity to promote phagocytosis of a given species of bacterium.

To eliminate the error due to incomplete mixing and settling out of cells during incubation, several authors have devised shaking and mixing machines which represent a definite advance in method. Soon after the publication of Wright's technique, Rosenow (58), in studying the comparative phagocytic effect of blood from normal persons and pneumonia patients, described a shaking machine operating in the incubator at 37°. With this apparatus he not only observed an increase in phagocytosis compared with that occurring in stationary preparations, but considered the results more reliable. By incubating and shaking mixtures of pneumococci and human defibrinated blood, he was able to demonstrate a definite pneumococcidal action of the latter which appeared to depend essentially upon phagocytosis and intracellular destruction, since normal serum, or that obtained after crisis from patients with lobar pneumonia, exhibited no bactericidal property in the absence of leucocytes.

Although shaking represents a definite advantage over stationary incubation, it does not permit the phagocytes to function at maximal efficiency in a given system, nor are the final phagocytic counts as uniform as when the mixture of leucocytes, serum, and particles is continually rotated in a sealed tube in a device such as that described by Fenn (59) and subsequently modified by Robertson and Sia (60), Todd (61), Ward (62), and others. When an apparatus of this general type is employed, reasonably accurate results may be secured, provided care

is taken to avoid certain sources of error such as those which may be introduced by suspensions of particles of unequal dispersion, or for the purposes of comparison, those which vary markedly in numbers. Agglutination of particles by serum constituents or by other physicochemical factors, also must alter the phagocytic count. As Neufeld and Rimpau (63) have shown, certain sera may cause the appearance of a prozone in which the number of particles ingested first increases with serum dilution and then decreases. Thus, particularly in cases in which the action of immune sera, or sera which have been long preserved, are the subject of study, it is not sufficient to use one serum dilution only. If the object of investigation be the effect of an induced physicochemical change upon the phagocytic capacity of the leucocytes themselves, such as variation in salt or hydrogen ion concentration, etc., in the event that serum be present, it is necessary to beware of interpreting any variation in the degree of phagocytosis as an indication of an alteration in leucocytic activity, since the change in the environment may have merely influenced the combining properties of the serum and the particle, thus leading to an increase or decrease in opsonization without in any way affecting the ingestive ability of the cell.

In examining the phagocytosis of bacteria it is of the greatest importance to have regard to the age of the culture. The organisms from young cultures may be very much more resistant to phagocytic attack than those examined after more prolonged incubation. This is well illustrated by the difference in behavior between young and old cultures of hemolytic streptococci as revealed by the studies of Hare (64, 65), Seastone (66), Ward and Lyons (67). With pneumococci the resistance of young cultures of certain types to ingestion by human leucocytes in normal homologous serum may be practically complete, while eighteen to twenty-four hour cultures yield organisms which are freely taken up by cells under the same conditions (68, 69). An accurate comparison of any experiments involving the phagocytic techniques cannot be made unless organisms of the same age are incorporated in the test. The newer knowledge concerning bacterial dissociation must necessarily be applied to phagocytic researches, since it is apparent unreliable findings will inevitably ensue if a culture containing organisms in more than one dissociative state is employed. When in the smooth phase a given bacterial species is in general decidedly more resistant to phagocytosis than when it has dissociated to the rough form. Organisms representing intermediate stages in the dissociative process may occur. It is therefore essential to make certain that a single form only is present in the suspension (70).

Not only have such mixing machines as we have referred to above, when used with the precautions which have been outlined, greatly

increased the precision of phagocytic measurements, but they have also permitted close estimations of the growth-inhibiting and bactericidal power of the serum-leucocyte mixture on such organisms as the pneumococcus or streptococcus, which are not subject to the lytic action of serum alone, but for their destruction require the participation of phagocytic cells. By the use of this method much has been learned through such researches, for example, as those of Robertson and his colleagues (60, 71, 72, 73, 74, 75, 76, 77), Todd (61, 78), Hare (64, 79, 80), Ward (62, 81), and Sutliff and Finland (82) concerning the mechanism of natural and acquired immunity to these species of bacteria.

In the animal body the degree of phagocytosis can be measured in a number of ways. An important method which has been employed by various workers (83, 84, 85) consists in determining the rate at which bacteria injected intravenously disappear from the blood stream. One accomplishes this by plating definite quantities of blood taken at intervals following the infection. This is a useful technique provided organisms such as the pneumococcus, streptococcus, or staphylococcus are used, which resist the lytic action of antibody and require the participation of phagocytic cells to bring about their destruction. The results obtained by blood culture may be supplemented by examining stained sections of such organs as the spleen, bone marrow, lung, and liver — the sites of the most active members of the reticular endothelial phagocytic system — for intracellular bacteria. Whether or not phagocytosis in these organs results in the destruction of the organisms may be learned by grinding weighed portions of tissue, emulsifying in physiological salt solution, preparing dilutions, and then plating definite quantities of these on suitable media.

The process of intracellular ingestion and even destruction of various microorganisms in the leucocytic exudates which appear within the various body cavities after artificial infection can be followed by either sacrificing the animal and washing out the cavity or by withdrawing small samples from time to time by means of capillary pipettes or hypodermic needles of small bore, preparing stained smears, and examining under the microscope (85, 86, 87, 88, 89).

Allusion (31, 32, 33, 34) has already been made to the use of tissue cultures in investigating the phagocytic behavior of various tissue cells. The results which have been obtained, although of great interest and possible significance, must, however, be interpreted with caution, since cells under these conditions may behave quite differently compared with their normal activity when growing in the organized tissues of the living animal.*

* For further references on the use of tissue cultures in the study of phagocytosis consult the review by Mudd, McCutcheon, and Lucké (90).

The Mechanism of Phagocytosis: Physicochemical Aspects. The factors governing phagocytosis in those simplified systems which can be established *in vitro* are numerous and complex. Within the living body the various constantly changing environmental circumstances which may influence the phenomenon obviously are even more difficult to analyze. In spite of these obstacles considerable progress has been made toward the understanding of the fundamental forces involved and of many agents which may modify their action.

In attempting to elucidate the mechanism of phagocytosis two stages may be recognized: in the first the cell comes in contact with the particle and in the second the particle is engulfed by the cell. We will begin by giving an account of the conditions, in so far as they are known, which promote contact between the cell and the organism.

Chemotropism. When the natural ameboid locomotion of the motile phagocytic cell is eliminated by rotation in sealed tubes in the manner alluded to in the previous section, collision between the two participants appears to be solely attributable to chance. Working with combinations of the polymorphonuclear leucocytes of the rat, carbon or quartz particles, and a suspending medium of isotonic sodium chloride solution and serum, Fenn (91, 92, 93) found that the rate of phagocytosis was proportional to the number of uningested particles. In addition to demonstrating the dependence of phagocytic rate on the number of available particles, he also showed that it was conditioned by the size of particle and cell and their relative velocities in the rotating tubes — evidence which further supported the conclusion that the probability of contact is due under these circumstances to chance alone.

But this is not the environment which in many sites in the animal body represents the theater of phagocytic activity, although it would seem that it affords a rather close analogy to the circulating blood. When a pathogenic bacterium enters the body, it frequently arouses an inflammatory response surrounding the point of entry, characterized by increased capillary permeability permitting the escape of leucocytes and the fluid constituents of the blood into the tissue spaces. Here these phagocytic cells "crawl" about by means of the pseudopodal extension and retraction of their cytoplasm. Under these conditions, which may be easily simulated *in vitro*, the contacts are most frequently occasioned not by casual meetings but by chemical attraction of some sort which is spoken of as chemotaxis. This may be either positive, *i.e.*, attractive; or negative, *i.e.*, repellent, depending on the nature of the material. A good example of the influence of the chemical nature of the particle in attracting the phagocyte is again an experiment of Fenn (93, 94), who allowed a suspension of cells and equal number of particles of MnO_2 and $MnSiO_3$ to run under a coverslip on a slide and observed the ensuing

phenomena under the microscope. With MnO_2, 2.4 times as many encounters took place as with $MnSiO_3$, resulting in the phagocytosis of 20 times as many particles of the former substance. This selective motion of the leucocytes toward these substances indicates a reaction on the part of the cell to changes in its environment set up by the particle. But this behavior as shown by the early work of Stahl (95) and Pfeffer (96) is by no means exhibited only by phagocytic cells but occurs under suitable conditions in the case of such a variety of unicellular organisms as myxomycetes, various infusoria, bacteria, and the spermatozoids of various plants. Leber (97, 11) in the course of his studies on inflammation was one of the first to examine the chemotactic properties of certain substances on leucocytes. He found that these cells were actively attracted by powdered copper and mercury compounds, but not by powdered gold or iron. Dead bacteria, he also observed, exerted a similar positive chemotactic influence, and Buchner (98) later succeeded in extracting from Friedländer's bacillus a protein exhibiting strong attraction for leucocytes. Moreover, the latter noted that glycine and leucine were definitely chemotactic, whereas tyrosine and trimethylamine were inert in this respect. It appears from these and other investigations that positive chemotaxis is an attribute of all bacteria, equally apparent in bacterial extracts or living and dead organisms. It is likely, therefore, that the attraction of leucocytes toward the point of invasion is in part, at least, due to the chemical properties of the bacterial aggressors. But the work of Massart and Bordet (99), who demonstrated the same migration of leucocytes in inflammations caused by bacteria indicated that this was probably not exclusively conditioned by the chemotactic effect of the microorganisms. Their findings showed that even products of leucocytic disintegration might be chemotactic. It would seem, therefore, that when many types of tissue injury occur, a stimulus results which attracts leucocytes. This accounts for the migration of these cells into inflamed areas not of bacterial origin, as well as local accumulation following the injection of insoluble inorganic substances. Lately Menkin (100, 101, 102), reinvestigating this problem, has isolated from sterile inflammatory exudates a crystalline nitrogenous substance revealing distinct chemotactic capacity in extremely small quantities. This material, tentatively termed "leukotaxine," the chemical nature of which has not yet been completely identified but which in the best preparations takes the form of needle-like crystals, also appears to be responsible for increased capillary permeability.

Various explanations have been advanced to account for chemotropism. Thus Maltaner and Hoppe (103) have asserted that osmotic forces govern the movement of phagocytes toward particles from which

dissolved substances are diffusing, since they observed that leucocytes enter capillary tubes only when they contain solutions of greater concentrations of certain materials than that of the medium in which the leucocytes are suspended. However, Wolf (104) has presented data which indicate that the chemotactic properties of a number of salts vary independently of the osmotic pressure of their solutions. Others (105) have considered the possibility that differences in the surface potential between the leucocyte and the particle or injured tissues might be sufficient to exert an attractive force between them. Abramson (106) and Feringa (107, 108) have suggested the possibility that emigration of leucocytes into areas of inflammation depends upon a difference in potential between the phagocytes and the injured tissue. But as Wells (109) points out, relatively high hydrogen ion concentration of the tissues which could induce a change in potential, cannot be the single determining factor in the migration of leucocytes since in certain conditions where this obtains, such as violent muscular activities or local asphyxia, no significant assemblage of these cells is observed.

There is, however, evidence from the studies of Menkin (110) that the hydrogen ion concentration plays a definite role in determining the prevailing type of cell in an inflamed area. The developing local acidosis conditions the predominating cell in the exudate, but apparently not through a chemotactic mechanism. Thus, this author found that when the *p*H of the tissue, such as in the earlier stages of inflammatory processes, is above 7, polymorphonuclear leucocytes are the most numerous among the cellular elements. With increasing hydrogen ion concentration attendant upon the progress of the lesion, the macrophage supplants the multi-nucleated cell. These researches indicate that the viability of leucocytes in exudates is a function of the hydrogen ion concentration. The polymorphonuclear cells are incapable of surviving in an acid medium. Mononuclear phagocytes, on the other hand, display perfect resistance in media at *p*H ranging from 7 to about 6.8. At greater hydrogen ion concentration these cells likewise succumb and frank suppuration results.

The hypothesis concerning chemotropism that most nearly seems to coincide with the majority of the facts would regard changes in the surface tension of the leucocyte brought about by soluble substances emanating from the particle or injured cells as the fundamental factors in the mechanism of the phenomenon. In considering the possible effect of surface tension in determining the motions of unicellular organisms, attempts have been made to imitate these by means of various lifeless systems. Bernstein (111), Rhumbler (112), and others produced "artificial amebae" which in almost all respects behaved like the living organisms by placing globules of mercury in acidified water containing

crystals of potassium dichromate. As the latter begins to dissolve, diffuses toward the mercury and touches it, the mercury globule will begin to become elongated and often move in the direction of the remaining undissolved dichromate. In addition, Rhumbler showed that a drop of clove oil in the presence of alcohol and glycerin becomes motile, and that a globule of chloroform in water will move toward a particle of shellac, flow about it, and dissolve it.

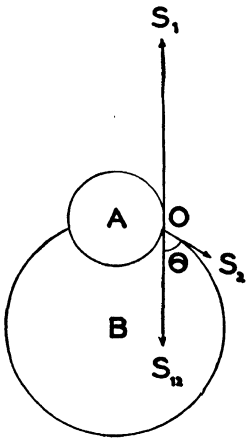
The similarity between these phenomena referable to surface tension alone and those taking place in living cells is therefore very striking. Wells (109, 113) has considered them at length and emphasizes the possibility of their relationship. He points out that positively chemotactic substances diffusing from a given direction and reaching the leucocyte will lower its surface tension on the side at which they come in contact, and there in consequence pseudopodia will be thrown out. The leucocyte will then move toward the point where these substances originate. Motion in a given direction will continue as long as the concentration of the chemotactic material is greater on this same side. When the concentration becomes equal on all sides, motion will cease.

The theory that surface changes determine the tropism of leucocytes, although conforming to many of the observed facts, has not been, as we have seen, universally accepted by all investigators. Furthermore, certain experimental findings more directly concerned with the actual ingestion of particles, which will be considered immediately, are admittedly difficult to account for on the basis of alteration in surface tension alone. We cannot conclude, therefore, that all the elements in the mechanism of leucocytic chemotropism have been entirely revealed, although there can be little doubt that surface relationships frequently play a major role.

Ingestion. The conditions underlying the second stage of phagocytosis, *i.e.*, ingestion, are also in part due, most probably, to the interplay of surface forces at the points of contact between cell and particle and their respective liquid interfaces. Fenn (94, 114) has formulated a relationship between the free surface energy at these interfaces when the system is in equilibrium which theoretically accounts for the mechanism of phagocytosis. This author has also derived the same equation more simply from a consideration of the forces of surface tension also under the condition of equilibrium. Mudd (90) has given a clear account of this latter formulation as follows:

Let O in the figure be a representative point in the line of contact between the three phases: fluid, particle, and phagocytic cell. Let the vectors S_1, S_2, and S_{12} be the interfacial tensions, respectively, in the particle-fluid, phagocyte-fluid, and phagocyte particle interfaces. It follows directly that at equilibrium $S_1 = S_{12} + S_2 \cdot \operatorname{Cos} \theta$, and either

contact between cell and particle will be maintained or partial ingestion of the particle will occur. If S_1 is greater than the two opposing forces, the surface of the phagocyte will tend to extend over that of the particle and complete ingestion will occur, whereas if S_{12} is greater than $S_1 + S_2$, neither ingestion nor adhesion will result.



Direct experimental support for the hypothesis is lacking, since no measurements of the interfacial forces under the three mathematically possible sets of conditions have been carried out. According to Mudd, however, it is possible to draw from the theory three deductions: (1) a quantitative correlation should exist between the degree of phagocytosis which may occur and the surface properties of the particles ingested, (2) phagocytosis is essentially a phenomenon of spreading of the cellular surface over the surface ingested, and (3) partial ingestion (or continued contact) should occur under certain circumstances. In a large series of experiments Mudd (115, 116, 117) and his co-workers have shown that the first deduction from the theory is largely upheld in phagocytic systems in which the surface properties of the particles (various acid-fast bacteria) are modified by the addition of normal or immune serum containing substances which promote phagocytosis. An account of these substances in animal blood sera will be presented subsequently in this chapter. These workers demonstrated that a constant correlation could be established between the changes in the surface properties of various bacteria induced by exposure to fresh unheated normal or immune sera and the readiness with which they were taken up by leucocytes. Such changes were revealed by agglutination, increased "stickiness" or cohesiveness, alteration in cataphoretic velocities, and in the ease with which "wetting" occurred as demonstrated by the behavior of the various kinds of particles at an oil-water interface. Increased phagocytosis went hand in hand with increased cohesiveness and "wetting" and varied inversely with the rate of migration in the electric field. Although particles tested differed greatly in their surface characteristics before exposure to sera, they afterwards exhibited a remarkable similarity. It was found that following treatment with homologous immune serum such diverse materials as acid-fast bacteria, sheep erythrocytes, and protein-treated collodion particles (118) all possessed characteristics in respect to cohesiveness and wetting comparable to that of denatured serum globulin, while their isoelectric points were in most cases also within the range shown by this substance. Further evidence for this close correspondence between the conditions at the surface and ease of ingestion is afforded by the experiments of Reiner and his co-workers (119, 120) and Freund (121) who noted that tannin altered the surfaces of bacteria and red blood cells and at the same time acted, as it were, as an artificial opsonin. Neufeld (122), Mudd (123), and Gordon (124) have subsequently analyzed this action of tannin and have found that it alters the aggluti-

nability in a manner entirely analogous to immune serum. Mudd has observed, however, that the electrical properties of the tannin-coated surface are markedly different from those shown by particles coated with serum. Gordon considers that substances possessing a tanning action form a non-ionized, non-hydrolyzable compound with the bacterial or erythrocytic proteins which presumably leads to changes in the character of the surface. In distinction to Reiner, he is not inclined to regard the known dehydrating effect of tannins as the essential factor in their capacity to increase phagocytosis.

Mudd (125) has also sought experimental support for the second deduction from the theory, *i.e.*, the surface of the leucocyte should spread over the particle. From direct microscopic examination he concluded that spreading of the cells occurs in many instances accompanied either by a marked distortion of the phagocyte or the flowing outward of hyaline protoplasm. Occasionally the particle may sink into the cell without marked deformation of contour. Never was ingestion within a vacuole of the suspending medium noted, which in itself strongly suggests that the process of ingestion is accomplished by an extension of the cellular periphery over the particle.

Experiment also supports the third deduction from the theory, since it has been shown that with insufficient sensitization (*i.e.*, insufficient change in the surface properties) in the presence of serum containing antibodies which promote phagocytosis, particles will adhere to the leucocytes but will not undergo complete ingestion. In the experiments of Bloom (126), using tissue cultures of rabbit lung and incompletely sensitized pigeon erythrocytes this condition was realized. When additional antiserum was added, prompt phagocytosis occurred.

In addition to the forces of surface tension, however, other factors undoubtedly enter into the complex phenomenon of phagocytosis and may then modify the effects which theory predicts. Of these the viscosity of the protoplasm of the cell is perhaps of foremost importance. This varies with temperature changes as well as spontaneously in local areas within the protoplasm where direct observation has detected reversible sol-gel transformations. At low temperatures Ledingham (127) found that particles stick to the cell but do not enter it. As the temperature is raised, changes in the internal state of the protoplasm take place which have been described by de Haan as characterized by alterations between sol and gel and attended by ingestion of the particles. Fenn (128) in attempting to define the temperature coefficient of phagocytosis, observed that at the point where these phenomena become marked, the quotient greatly increases. Finally it must be borne in mind that the living cell is not like the mercury or oil droplet but represents a more or less definite structure within which the energy changes resulting from metabolic processes continually take place. These may be of sufficient magnitude to alter or suppress the forces of surface tension. It is highly probable, however, in the light of the evidence afforded by the extensive studies of Fenn and Mudd, that these

forces represent an important factor in phagocytosis by leucocytes and presumably by the other phagocytic cells of the metazoan body.

The Effect of Certain Physical and Chemical Agents on Phagocytosis. Many studies have been carried out directed toward analyzing the effect on phagocytic systems of various chemical compounds and of purely physical entities such as heat, osmotic pressure, and ultraviolet irradiation. Space does not permit us to review these results in detail, and the interested reader is referred to the comprehensive summaries of Hamburger (129) and Mudd (90). Here it must suffice to describe briefly the action, so far as it is known, of only a few of the more important of these agents.

Madsen (130, 131) and his associates and Fenn (128), among others, have studied the effect of increasing temperature on the phagocytosis of bacteria (staphylococci, B. coli) and inert particles (carbon and quartz). Although both investigators found, as was indeed to be expected from the experiments of earlier workers such as Ledingham (127), that the number of particles ingested within a given time increased with rising temperature, mathematical treatment of their data by Fenn with a view to deriving a temperature coefficient yielded conflicting results. We are therefore unable to state precisely the effect of temperature in general quantitative terms. In actual experimental work a constant temperature of 37° C. is usually maintained when mammalian phagocytes are employed. It is possible, however, that in cases where the greatest accuracy is required or analogies are to be drawn between findings *in vitro* with phenomena which may occur *in vivo* that the temperature should be adjusted to reflect that of the normal or diseased body of the particular species of animal from which the leucocytes and serum were derived. For Madsen and Wulff (130) found that optimum phagocytosis of human, guinea pig, chicken, and pigeon leucocytes occurred at temperatures corresponding to the normal for these species. Moreover, it was noted that if the temperature of the individual varied, maximum phagocytosis was obtained at a temperature equivalent to that exhibited by the individual at the time the cells were collected.

Changes in temperature may influence the different components of the phagocytic system in various degrees. Thus at relatively low or high temperatures the activity of the leucocytes is greatly altered, while in the intermediate range the condition leading to combination between particle and the sensitizing constituents of the serum may be chiefly affected as Ledingham (127) has shown. It is, therefore, perhaps not surprising that the results of various authors, working with different sorts of particles, leucocytes, and suspending media, frequently do not coincide in their details.

A few observations have been recorded on the changes induced in

phagocytic systems by variations in osmotic pressure relationships. Wright and Reid (132) found that moderately hypotonic sodium chloride solutions increased the ingestion of tubercle bacilli by human leucocytes. With further decrease in salt concentration (below 0.6 per cent NaCl) phagocytosis decreased. Similar findings have been recorded by Ouweleen (133). In contrast, Hamburger (134) asserts that maximal effect is obtained with solutions isotonic with the blood. All these investigators are agreed that hypertonic sodium chloride tends to inhibit the reaction.

Because of the apparent favorable influence of ultraviolet and X-ray irradiation on certain infections, a number of experiments have been undertaken with the object of discovering whether these clinical findings might be related to stimulation of the phagocytic defense mechanism. The literature, which is to a certain extent conflicting, has been summarized by Fleischmann (135). There seems to be no conclusive evidence to indicate that ultraviolet irradiation enhances either the activity of leucocytes or the sensitizing capacity of serum. Indeed, on the whole, the results indicate that both elements may be injured thereby. On the other hand, preliminary exposure of bacteria such as staphylococci to ultraviolet light does seem to markedly increase the ease with which they are taken up by cells.

The effect of various ions on phagocytosis has been studied by a number of investigators. The results for the greater part are not of great interest to the immunologist. Of practical value, however, are the findings of Hamburger (134) concerning the depressing effect of the citrate ion, and the action of Ca in restoring the phagocytic powers of leucocytes which had been reduced by exposure to isotonic NaCl for 24 hours. The observation on citrate is important, since it contraindicates the use of citrated blood in phagocytic experiments. Of importance also are the reports of Hamburger (134), Evans (136), Fenn (137), and others on the influence of changes in hydrogen ion concentration. Their experiments, taken as a whole, indicate that optimum phagocytosis occurs at about neutrality or in some cases slightly on the acid side. Any marked increase or decrease in the *p*H brings about definite diminution in the phagocytic index. No adequate explanation for the influence of *p*H on phagocytosis has been presented, although it would seem *a priori* most likely that the leucocyte may be injured by increases in hydrogen or hydroxyl ions. Mudd (90) has suggested that changes in *p*H may affect the combining properties of serum constituents and particles.

Still other classes of known chemical substances have been tested, such as the fat-soluble organic compounds, iodoform, chloroform, chloral hydrate, and ethyl alcohol, histamine, dextrose, certain drugs

such as arsphenamine, and the lipoidal substances lecithin and cholesterol. In many instances conflicting results have been obtained by various workers. For details the interested reader may consult the works of Hamburger (129, 134), Fleischmann (135), Mudd (90), and Tunnicliff (138).

Bacteriophage, which according to the latest statement of Northrop (139) probably is of the nature of a nucleoprotein, was found by d'Herelle (140) to increase the susceptibility of bacteria to phagocytosis. Nelson (141) has studied this phenomenon in detail, using staphylococcus and its specific phage. With resistant organisms or heterologous phage no increase in the phagocytic index was observed, which increased ninefold, however, when the specific system was employed. Gerards (142) has shown that following the injection of phage into a rabbit the phagocytic index for the leucocytes and serum of that animal is markedly increased even after a period of seven days. The phage seems to affect both the bacteria and the cells, so that the former become more susceptible to phagocytic attack and the latter more active in ingestion (90). This is certainly a very important property of phages and may well receive more investigation than it has in the past.

Humoral Factors Determining Phagocytosis — The Opsonins of the Blood Serum. We have seen that phagocytosis is a process which largely depends upon the surface properties of the particle in relation to those of the cell. Substances enhancing ingestion effect changes in the surfaces in the direction of increasing the tendency for cell and particle to adhere and for the latter to merge into the cell. Of those substances which are known to have this effect certain constituents of normal and immune serum are the most potent. As we shall see, these are in most cases antibodies and in certain instances have been identified with the precipitins and agglutinins which have already been described.

From the beginnings of his researches upon phagocytosis Metchnikoff recognized that the process was influenced by the blood plasma in which the phenomenon occurred. Both he and Bordet (143) noticed that the phagocytic activity of leucocytes was greater in immune than in normal sera and associated this with the specific properties of the immune substances or antibodies. Metchnikoff himself interpreted the phagocytosis-enhancing power of the serum as a stimulation of the leucocytes and referred to the serum constituents by which this effect was produced as "stimulins." A closer analysis of the factors involved in this relationship, however, was not attempted at this time, although indirect reference was made to it in a number of articles emanating from his school.

The first investigation which occupied itself directly with these problems was that of the Belgian bacteriologists Denys and Leclef (53). The publication of these workers deals with the nature of streptococcus

immunity in rabbits. It established, first, the decisive importance of phagocytosis in the resistance of animals against these bacteria, and made clear that the destruction of bacteria was carried out as well by the leucocytes of normal as by those of immune animals, but was powerfully enhanced when the leucocytes of either a normal or immunized animal were combined with immune serum. Their work, therefore, indicated that the increased phagocytosis of virulent bacteria, taking place in immune animals, depended upon alterations in the functions of the serum rather than in those of the cells. They suggested, furthermore, that the influence of the serum was not one of leucocyte stimulation, but acted upon the bacteria, rendering them less resistant to phagocytosis. They say in substance: "A notre avis, on pourrait tout aussi bien admettre que la substance vaccinante ou antitoxique agit, non pas sur le leukocyte, mais sur un poison renfermé dans le corps du microbe ou dissous dans le milieu, et qui préserve le microörganisme contre les atteintes du leukocyte." *

Observations with streptococci and pneumococci carried out after this by Marchand (144) and by Mennes (145), besides confirming most of the observations of Denys and Leclef, brought out especially the relation of the virulence of microorganisms to phagocytosis, showing that very virulent strains were taken up to only a slight degree in the presence of normal serum, but were subject to active phagocytosis when immune serum was employed. Avirulent strains on the contrary were rapidly phagocyted in normal fresh serum. This, too, seemed to point to the fact that the serum influenced the bacteria rather than the phagocytes, although no conclusive proof is brought for this in their publications. No definite progress was made beyond the results of Denys and his pupils until 1902, when Leishman (55) introduced a technique by means of which it became possible to observe the process of phagocytosis with fresh serum and leucocytes *in vitro*.

By utilizing this technique and improving upon it, Wright and Douglas (146) in the following year, as we have seen, evolved a method by means of which phagocytic activity could be quantitatively measured. They worked at first with staphylococcus phagocytosis by human leucocytes in the presence of human citrate plasma, a research undertaken primarily

* In our opinion one can just as well believe that the vaccinating or anti-toxic substance acts not upon the leucocyte but upon a poison enclosed within the body of the bacteria or dissolved in the medium, which preserves the microorganism against the attacks of the leucocyte.

Denys formulated this view with still greater clearness and positiveness at the Congress of Hygiene held at Brussels in 1903. We take our citation from the discussion on opsonins by Gruber (3d meeting Freie Vereinigung f. Mikrobiol., Vienna, 1909, *Centralbl. f. Bakt.*, I Ref., Vol. 44, Suppl. p. 3). Following is Denys' statement: 1. The phagocytosis in immune sera is dependent upon substances which are precipitated with the euglobulins. 2. These substances cause phagocytosis by inciting a physical alteration of the microorganisms. 3. These substances are specific.

because Wright (147), in collaboration with Windsor, had previously determined that human blood serum possessed practically no bactericidal power for this organism, and that phagocytosis was the chief mechanism of protection against these bacteria. The researches of Wright and Douglas extended the observations of Leishman and established the fact that normal sera contained substances which promoted the phagocytosis of a large variety of bacterial species. For these elements they coined the term "opsonins" (*ὀψονέα* = I prepare food), since they found that they reacted in some manner with the bacteria, preparing them for ingestion by the leucocytes. Although not entirely excluding the possibility of serum factors acting as "stimulins" in the Metchnikovian sense, they concluded that if they existed, they were of relatively little significance in the phagocytic mechanism. This conclusion was based upon experiments in which bacteria and leucocytes were treated separately with fresh serum, washed, and then brought together. It was found that leucocytes exposed to serum and then washed exhibited no increase in their capacity to take up bacteria suspended in salt solution. In contrast organisms treated with serum, washed and resuspended in 0.85 per cent salt solution were readily taken up by the washed leucocytes. Hektoen and Ruediger (148) soon confirmed their results, and from their paper we take as an illustration the modified experimental protocol which follows:

PHAGOCYTOSIS OF SENSITIZED BACTERIA BY HUMAN LEUCOCYTES

	Phagocytic Index
Leucocytes + fresh normal human serum + *Staphylococcus aureus*	22
Leucocytes washed in saline + *Staphylococcus aureus* + saline	1.2
Leucocytes washed in saline + *Staphylococcus aureus* treated with fresh normal human serum and washed in saline + saline	10
Leucocytes + fresh normal human serum + streptococcus	22
Leucocytes washed in saline + streptococcus	1
Leucocytes washed in saline + streptococcus treated with fresh normal human serum and washed + saline	14
Leucocytes washed in saline + streptococcus treated with fresh normal guinea pig serum and washed + saline	12
Leucocytes washed in saline + streptococcus treated with fresh normal rabbit serum and washed + saline	14

In agreement with the work of Denys and his pupils, Leishman (55) first, and then Wright and Douglas (146, 149, 150), made the important observation that the opsonic indices of patients suffering from various localized infectious processes increased following the injection of killed vaccines. Their method of therapeutic vaccination was developed as a result of these findings.

A third fundamental fact was revealed by their work. They noted that the opsonic effect of normal sera was in many, although, be it said, not in all instances, greatly reduced or even obliterated by heating the serum at 55°–60° for 10 minutes or longer. Exposure to light and preservation for relatively short periods of time in sealed tubes likewise resulted in marked diminution or extinction of this property. A protocol from Wright and Douglas's first paper (146) will illustrate the degree of reduction of opsonic power resulting from the exposure of normal serum to 60° for 10 to 15 minutes:

A. Unheated serum Wright—Staphylococcus suspension 1 vol.—Blood cells Wright 3 vols.
   (1) Phagocytic average 20 cells..... 17.4
   (2) Phagocytic average 20 cells..... 19.8
B. Heated serum as above.
   (1) Phagocytic average 52 cells..... 0.6
   (2) Phagocytic average 46 cells..... 3.4

Because of this lability of the opsonin, Wright at first was inclined to believe that phagocytosis was impossible in the absence of serum. However, it soon became evident through the work of Löhlein (151) and many others that "spontaneous" phagocytosis could take place in the absence of serum of any kind. Spontaneous phagocytosis does not occur with all particulate substances. In the cases of many bacteria it appears to be governed by their state of virulence, although this is not an invariable rule. Rough pneumococci, for example, are appreciably phagocyted by washed human leucocytes in contradistinction to the S forms, as indeed are many other avirulent variants of pathogenic organisms. Wright, who soon realized his error, with Reid (132), studied the conditions governing spontaneous phagocytosis particularly in respect to concentration of electrolyte. They found that this was maximum at about 0.6 per cent NaCl and diminished with increasing salt. When the concentration attained 2.7 per cent, spontaneous phagocytosis of tubercle bacilli ceased.

In Wright's early work there is nothing explicit concerning the specificity of the opsonic substances in normal serum. It can be inferred, however, from the fact that the opsonic power of the same serum against different species of bacteria was found to be variable.

The problem of the relationship of opsonins of normal sera to those appearing as the result of active immunization was not analyzed by Wright and his collaborators. Following the lines laid down by Denys and his collaborators with bacteria, and by Sawtchenko (152), who showed that substances which enhanced phagocytosis appeared in the blood of animals injected with foreign erythrocytes and that these substances were specific in their effect, Neufeld and Rimpau (153, 154)

demonstrated that the opsonic properties of antistreptococcus and antipneumococcus sera were thermostable like other immune bodies, which produce agglutination, precipitation, and bacteriolysis. These thermostable opsonins were shown, like those of Sawtchenko, to be specific in their action — which in itself, of course, was further proof that they did not act upon the leucocytes. Neufeld called these antibodies bacteriotropins. These Neufeld held to be simple factors comparable to precipitins or agglutinins which did not require to produce their maximum effect the adjuvant action of any thermolabile constituent of the serum such as is necessary in the case of hemolytic or bacteriolytic antibodies. He subsequently observed, however, that heating some, but not all, immune sera at 55°–60° C. did reduce their capacity to induce phagocytosis, which could be restored, by the addition of a little fresh normal serum, itself exhibiting slight or no opsonic effect. Accordingly he postulated the existence of a second specific immune opsonin analogous to the "amboceptor of Ehrlich." In the years which followed, these observations and the various hypotheses based upon them led to much discussion, confusion, and experimentation. More recently, due to the development of techniques by which relatively pure antigenic substances have been prepared from bacteria, it has been possible to arrive at a more simple conception of the nature of these apparently diverse serum constituents concerned in the mechanism of phagocytosis. But before these later results can be properly assessed, we must briefly review the significant data revealed by earlier investigators in their efforts to illuminate this complex subject.

In their simplest terms the questions at issue have been:

(*a*) Is the opsonin of normal serum an entirely new sort of antibody?

(*b*) Is normal opsonin identical with complement?

(*c*) Is normal opsonin dependent upon the combined action of two factors:

(1) a thermostable specific antibody and

(2) complement or alexin; and if so, is the specific element identical with that found in the serum of immunized men and animals?

(*d*) Do two qualitatively distinct thermostable specific immune bodies exist as Neufeld believed, or can the observed differences be explained on quantitative grounds?

In their earliest investigations Wright and Douglas regarded the normal opsonins as new antibodies separate and distinct from those already known. Time has afforded little evidence in support of this theory, and it is therefore not necessary to discuss it here in any detail.

The opinion that normal opsonin and complement might be the same was suggested by many similarities in their behavior. We have seen

that like complement, opsonin is largely inactivated in a few minutes at 55°–60° C. Moreover, in a further analysis of their physical properties Noguchi (155) found that they both resisted drying at 23° C. and in this state would withstand a temperature of 135° C. without much loss of activity. Von Dungern (156) as early as 1900 showed that various substances such as bacteria, organic and inorganic particles absorbed opsonin from serum just as they did complement. Muir and Martin (157) found that all antigen-antibody complexes which absorb complement out of serum at the same time remove the normal opsonin. Thus sensitized red blood corpuscles, sensitized bacteria, and specific precipitates added to normal serum remove its opsonic properties. From this fact they also concluded that the normal opsonins like alexin were non-specific. For just as the alexin of a serum may serve to activate a considerable variety of sensitized antigens, so the opsonin of normal serum, they stated, may unite with a large number of different sorts of particles. Similar observations were recorded by Neufeld and Hüne (158), who noted that yeast cells will absorb both complement and opsonin. Levaditi and Inman (159) and Baecher (160) presented further evidence on the similarity of behavior of these two constituents of normal serum. The former, for example, showed that whereas both are absent from the anterior chamber of the normal eye, they both simultaneously enter the aqueous humor after injury. A like conjunction in their presence or absence has been shown for edema fluids. Also phosphorus poisoning which reduces complement likewise reduces opsonin.

Although this parallelism is striking, it obviously does not mean that the two are necessarily identical. It may signify merely that the alexin or a factor closely resembling it is a participant in normal opsonic action just as Dean (161) and Neufeld and Bickel (162) showed that it was in certain cases where immune opsonic substances were concerned, and that it activates a thermostable specific constituent in a manner analogous to, but not identical with, that in which it activates hemolytic or bactericidal sensitizors. This conception has been expressed by Dean (163) and others, and indeed it appears to afford not only the most logical view but one which agrees most closely with the results of experiment. For the procedures which remove both complement and opsonin, as stated above, do not always, as a matter of fact, remove *all* the opsonic action. Studies of Hektoen as well as of Wright himself, as will be seen from the protocols given above, have proved that although usually markedly reduced, nevertheless the opsonic capacity of heated normal serum is quite definite compared with salt solution. A similar remnant of opsonic action after absorption of normal serum with sensitized cells, bacteria, and precipitates is evident from the experiments of Muir and

Martin (157). The significance of this point becomes clear when we consider the properties of the bacteriotropins which are heat stable and capable of opsonizing in the absence of complement. It would seem, therefore, that there is frequently present in normal serum a slight amount of thermostable opsonin which, though capable of acting feebly by itself, is, nevertheless, powerfully increased in its effect by complement. Direct evidence for this view is afforded by the experiments of Cowie and Chapin (164), who were able to almost completely restore the profoundly diminished opsonic properties of heated normal serum by the addition of a small amount of fresh serum which by itself produced only a minimal degree of phagocytosis. The details of one of their experiments are given in illustration to this effect:

	Phagocytic Index
1. Unheated serum	15.44
2. Salt solution	0.18
3. Heated serum, 57° C.	1.08
4. Diluted serum (1 : 15)	1.56
5. Heated serum 57° C. + diluted serum (1 : 15)	12.40
6. Unheated serum + diluted serum	16.08

A number of other workers such as Dean (161) with normal rabbit's serum and typhoid bacilli, and Eggers (165) with avirulent pneumococci have obtained comparable effects. In this type of experiment, however, it is not always easy to secure positive results. One of the present authors with Ward (68) employing Cowie's technique failed to reactivate by means of diluted serum heated normal human serum which was, in the fresh state, strongly opsonic for virulent pneumococci (Type I), although a thermostable opsonin could be demonstrated provided sufficient complement was added in the form of undiluted fresh infant's serum, which by itself had practically no effect. We believe it is probable that negative results such as those of Fornet and Porter (166) are due to neglect of such quantitative considerations.

Most of those who have studied this problem have found that the opsonins of immune sera behave in an essentially identical manner. Thus Dean (161), using a heated immune serum which alone revealed considerable activity, demonstrated a marked increase in its capacity to promote the ingestion of typhoid bacilli when small amounts of fresh normal serum feeble in opsonin were added. Hektoen's (167) experiments with hemopsonic immune sera are analogous, as likewise are those of Neufeld and Bickel (162) and Levaditi and Inman (159). By 1909, then, there was much reason for believing that the full opsonic action both of normal and immune sera is dependent upon the co-operation of a thermolabile and a thermostable constitutent and that the mechanism of normal and immune opsonins, after all, differs only in quantitative

relations between the two. Complete acceptance of this conception was prevented and is even now made difficult in some cases by the problem of the specificity of normal opsonin, to which allusion has already been made. For if, as at first supposed, this factor is, like complement, non-specific, the amboceptor complement hypothesis would be rendered unlikely. Earlier work upon this question yielded contradictory results. Bulloch and Western (168) with staphylococci and tubercle bacilli found that each of these organisms absorbed out separately specific opsonins from normal serum, leaving those for other bacteria but slightly reduced. On the other hand, Simon, Lamar, and Bispham (169), Russell (170), Axamit and Tsuda (171), and a number of others failed to find evidence of such specificity, in that a variety of bacteria seemed to absorb opsonins out of normal serum indiscriminately without giving any indication of selective action. But the careful work of Rosenow (172), Macdonald (173), and Hektoen (167) upheld the original contention of Bulloch and Western (168). Later Bull and McKee (174) demonstrated type-specific pneumococcal protective substances in normal chicken serum. These are almost certainly capable of opsonic action. Sia (175), in 1927, working with the normal sera of the cat, dog, sheep, and pig, by absorption experiments revealed the presence of opsonins specific for various types of pneumococci. In our own hands (68) the removal of type-specific pneumococcal opsonins from normal human sera by absorption with bacterial suspension has proved more difficult. Delicate adjustment of the amount of the absorbing suspension was an essential factor, and all operations of absorption had to be carried out at about 4° C. When these precautions were taken it was possible, however, to demonstrate a selective removal of opsonin according to the type of organism employed. Moreover, this was performed with practically no reduction in the hemolytic complement present in the normal serum. Further evidence for the nature of normal opsonin is to be found in the experiments of Sia (74) and of Ward (81) on the type-specific inhibition of the bactericidal power of serum-leucocyte mixtures or human defibrinated blood by the soluble specific substances of Pneumococcus Types I, II, and III. Since it is known that the destruction of the pneumococcus in such systems takes place only through phagocytic co-operation, it was apparent that the addition of the type-specific carbohydrate had specifically inhibited the normal homologous opsonin, although this was not directly demonstrated by means of phagocytic counts on stained preparations. Later complete demonstration was afforded by the experiments of Ward and Enders (68), who found that following the addition of the specific carbohydrates of Pneumococcus Types II and III, the ingestion of young encapsulated cultures of pneumococci by the polymorphonuclear leucocytes in fresh normal adult human blood was specifically

inhibited. The Pneumococcus Type I carbohydrate did not exhibit an effect so pronounced probably because the deacetylated form of the specific carbohydrate was employed, since subsequently it was shown by Enders and Wu (176) that the acetyl polysaccharide did practically eliminate the opsonic action of human sera. These normal opsonins were shown by Ward and Enders to be thermostable inasmuch as their activity, which had been impaired by heating at 56° C. for 30 minutes, was restored by the addition of fresh infant's serum which by itself was incapable of exerting any significant opsonic action, although it contained a large quantity of hemolytic complement. Furthermore, it was observed that such heated normal sera could lead to phagocytosis of a considerable number of organisms even in the absence of fresh serum, provided contact between cocci and serum was maintained for 24 hours prior to the addition of the cells. In other words, it was demonstrated that the normal serum could act as a tropin in the sense of Neufeld. This tropic action was again specifically checked upon the addition of soluble specific substance.

While the specific action of normal opsonins has been thus made evident in numerous instances, it must, however, be recognized that normal serum, particularly when it is unheated, enhances the phagocytosis of various inorganic and organic particles, the nature of which makes it difficult to understand how a specific antibody could be involved. Thus such substances as carbon, flour, starch granules, and quartz particles are much more readily ingested following exposure to normal serum (169, 177, 178, 179). Possibly here the properties of the surfaces are such that the normal serum proteins are easily adsorbed, after which they may undergo denaturation or other change analogous to that which takes place when antigen unites with antibody. The physical attributes of denatured protein seem to render it particularly suitable for ingestion by phagocytes. In this manner the phagocytosis of avirulent bacteria, such as dissociative forms and saprophytic varieties, might also take place, although the participation of natural antibody should be eliminated in any specific instance before we can assume that the opsonization is not specific.

In view of all the foregoing experimental evidence we may conclude that the opsonic power of normal serum *when it is specific* usually depends upon the co-operation of two components, (*a*) thermostable antibody and (*b*) a thermolabile substance similar to complement. The first component can by itself opsonize, but this effect is most often greatly enhanced by the presence of the second, which, however, is alone incapable of inducing phagocytosis.

Can we, now, extend this well-established conception of the opsonic mechanism to cases in which immune sera are concerned? It is uni-

versally agreed that the opsonic antibodies of such sera are both strictly specific in their action and resistant to heating for 30 minutes at 55° C. They appear then to be analogous to, or identical with, the thermostable specific component of normal serum. Moreover, as we have observed, the early work of Dean, Hektoen, and others showed that the activity of the opsonic antibody of immune serum could be increased by the addition of a small amount of fresh normal serum. But Neufeld* denied that this complementing effect could be always demonstrated, although freely admitting that in many instances it does occur. To explain this apparent discrepancy he postulated, as we have seen, two different kinds of opsonic antibodies both of which may be found in both normal and immune serum: the tropins acting entirely without the aid of the thermolabile factor, and the opsonic amboceptors which require its presence before exerting any effect. From the beginning this seemed to many an unnecessary multiplication of entities, but it was difficult to devise a conclusive experiment. As with the normal opsonins, the discovery and isolation of the type-specific soluble specific substances of the pneumococcus made it possible to put these conflicting theories to experimental arbitration. Employing young encapsulated pneumococci, type-specific antisera, and the polysaccharides of Pneumococcus Types I, II, III, Ward and Enders (68) established the following facts:

(1) The opsonic action of heated immune sera alone is eliminated by the addition of the homologous polysaccharide.

(2) The opsonic action of diluted antiserum is increased by the presence of fresh normal serum, itself incapable of inducing phagocytosis.

(3) The opsonic action of the diluted antiserum in the presence of fresh normal serum is also eliminated by the addition of the homologous polysaccharide.

Since in these cases, just as in those with normal opsonins which have been previously cited, we are dealing with a single antigen which presumably reacts with a single antibody, it seems justifiable to conclude that one and the same antibody can be responsible for the specific phagocytic action of normal unheated and heated serum, inactivated immune serum, and immune serum activated by fresh non-opsonic normal serum.

This demonstration that the type-specific anticarbohydrate antibody can be the sole antibody concerned in the phagocytosis of virulent pneumococci definitely relates the phenomenon to the other serum reactions, since it is known that this agglutination, precipitation fixation of hemolytic complement, and anaphylaxis may all occur as a result of the union under suitable conditions of this antibody with its homologous antigen.

It is hardly necessary to point out that these findings are in entire

* For a summary of Neufeld's views see his chapter on Phagocytosis in Kolle and Wassermann's *Handbuch* (180).

accord with the "unitarian" conception of antibodies formulated by Dean (181) and Zinsser (182).

Thus we have arrived at a simple explanation for the opsonic mechanism wherever it is governed by specific serum factors irrespective of whether these are associated with normal or immune serum. A single antibody, capable of bringing about agglutination, precipitation, and other immune reactions, may also prepare the bacterium or other particle for ingestion by uniting with the antigen exposed at the surface, thereby altering the surface properties in such a manner that they come to resemble those of denaturated serum globulin. The efficiency of the antibody in promoting this effect is greatly increased by a substance resembling hemolytic complement unless relatively very large quantities of the antibody alone are employed.

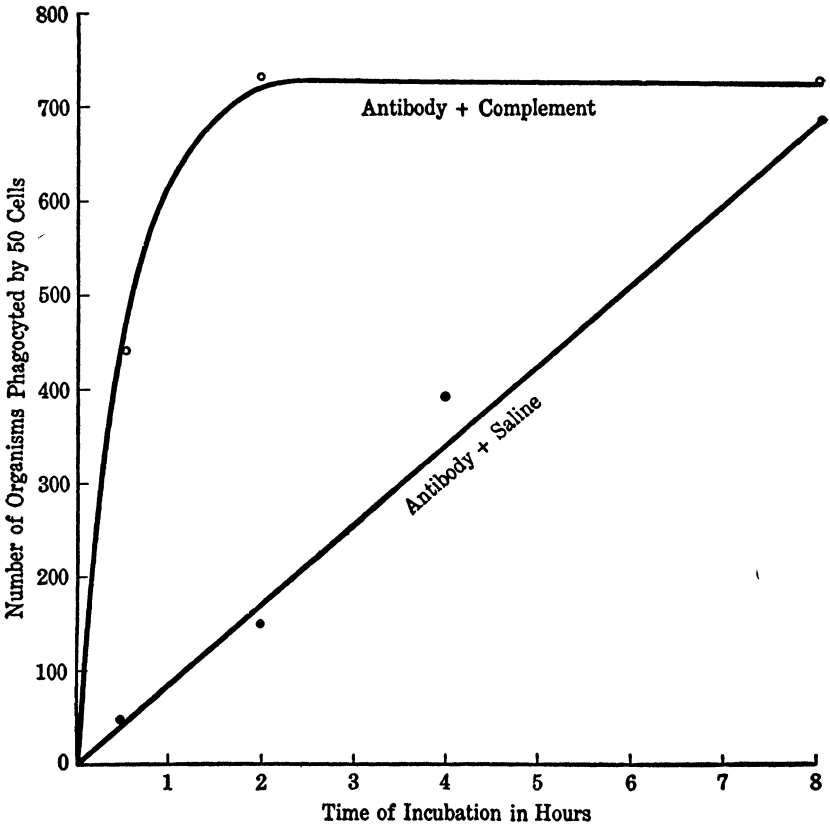
It remains to inquire whether this thermolabile factor is identical with the complement which is essential in the hemolysis of red blood cells and to present what is known as to how it acts in conjunction with opsonic antibody to enhance the effect of the latter.

We have referred to the experiments of Muir and Martin (157) and others which showed that both hemolytic complement and the opsonic properties of normal serum were removed by the addition of many kinds of finely divided matter. The thermolability of both complement and the non-specific opsonic factor in serum is the same. Dilution in water, as shown by Browning (183), destroys both complement and the opsonic properties of normal serum. Early experiments, however, by Ledingham (184) and Zinsser and Carey (185) on the effect of the albumin and globulin fractions of complement in phagocytic systems suggest that the thermolabile constituent of opsonin is not identical with complement, although closely associated or identified with portions thereof. More recently Gordon and his associates (186) have demonstrated that the so-called "fourth component" of complement discovered by them in 1926 is not necessary for opsonization, although essential in hemolysis and bacteriolysis. This component is destroyed by treating the fresh serum with ammonia.

We cannot conclude, therefore, that hemolytic complement and the thermolabile substance which co-operates with the antibody in phagocytosis are identical, although they resemble each other in many respects.

As far as we know from the insufficient experimental work which has been recorded, one of the possible functions of the thermolabile factor in specific opsonization is that of a catalyst which increases the speed of combination between antibody and antigenic particle. Evidence for this is afforded by the early work of Wright and Douglas (146), followed by that of Bulloch and Atkin (187) and of Sellards (188), which showed that if bacteria were exposed to the action of unheated normal serum and

subsequently heated to 60° C., they were phagocyted almost as readily as organisms subjected simply to the unheated serum. The probable explanation of these results seems to us to lie in the rapid union of the available antibody with the organism under the influence of opsonic complement. More direct evidence was secured by Ward and Enders, who determined the rate of phagocytosis of heat-killed encapsulated



PHAGOCYTOSIS WITH AND WITHOUT COMPLEMENT

pneumococci in the presence of an appropriate quantity of inactivated and diluted homologous antiserum with and without the addition of unheated normal serum. Phagocytic counts were made at intervals over a period of 8 hours. The results are included in the figure above. It would seem that the thermolabile factor under these conditions does not increase the absolute capacity of antiserum to promote phagocytosis but merely appears to do so by accelerating the velocity with which the organism is prepared for ingestion by the cells through its union with the

anticarbohydrate antibody which in this case was the only specific factor concerned. In normal serum where the quantity of thermostable opsonin is small, it is quite evident that the accelerating effect of the thermolabile factor is extremely important under the relatively brief reaction time usually allowed in phagocytic work. In this connection it is of interest to recall the observations of Rosenthal (189), who found that the opsonic effect of heated normal serum took place more slowly than when complement was present.

Opsonins in Relation to Macrophages and Other Phagocytic Cells. Our knowledge of the nature and mode of action of the phagocytic process is almost entirely based upon experiments involving the use of polymorphonuclear leucocytes. The question arises as to whether other types of cells, particularly the macrophages — both wandering and fixed — behave in the same manner toward bacteria which have been altered by the opsonic antibody. The literature on this subject is not extensive but it seems to be conclusive. As early as 1907, Briscoe (190) showed that *in vitro* heat-stable opsonins definitely increased the phagocytosis of various substances by macrophages. Numerous investigators, including Briscoe, Kitagawa (191), Singer and Adler (192, 193), Bass (194), Tsuda (195), Wright (196), and Cannon and Pacheco (197), have observed the more rapid and permanent disappearance of bacteria injected into both actively and passively immunized animals in comparison to normal controls. In many of these cases cultural and histological examination of the organs indicates unequivocally that the organisms have been ingested by the cells of the reticulo-endothelial system. Possibly even more direct evidence that these cells are stimulated to increased activity toward opsonized bacteria is afforded by the work of Manwaring (198, 199), who perfused livers and spleens with suspensions of bacteria (pneumococci) which had been exposed to specific antiserum and found that these were retained in the organs in contrast to organisms treated with normal serum.

In vitro studies on tissue cultures containing macrophages from the spleen were employed by Loewenthal and Micseh (200) in analyzing the effect of pneumococcus antibody in promoting phagocytosis. They found that virulent pneumococci resistant to phagocytosis without the presence of type-specific antiserum were readily ingested following its addition to the culture flask. Bloom (201), also using tissue culture methods, observed a similar increase in the ingestion of red blood cells by macrophages after opsonification with specific antiserum. Finally Mudd (90, 202, 203) and his associates found nearly a complete agreement between the phagocytic behavior of exudative polymorphonuclear leucocytes and macrophages toward a variety of bacteria sensitized with fresh and heated normal and immune sera. The only significant differ-

ence was in the phagocytosis of collodion particles and oil droplets by macrophages which were refused by the polymorphonuclear cells.

In addition to establishing the fact that opsonins, either normal or immune, prepare bacteria and other particulate antigens for phagocytosis as well by macrophages as microphages, these data indicate that Metchnikoff's original conception of the latter as the phagocyte *par excellence* against most bacteria cannot be maintained.

Fluctuations in Phagocytic Capacity of the Cells. In our review of the chemical and physical factors which may influence phagocytosis, we have alluded briefly to the effect of certain of these upon the phagocytic cells which may alter their ingestive capacity. We have seen that changes in temperature, in concentration of ions and the presence of fat solvents and bacteriophage may act upon these cells. It should be observed, however, that differences in the phagocytic energy of leucocytes may vary, depending upon the individual source whence they are derived (204, 205).

Rosenow (206) and Tunnicliff (207) have shown that the inherent phagocytic power of leucocytes may vary not only from one individual to another, but also in health and disease. Madsen and Wulff (130), it will be recalled, found the optimum temperature for leucocytic activity to correspond to that existing in the body at the time the leucocytes were withdrawn. Age appears to be a factor in determining the activity of these cells, for Tunnicliff showed that at birth they were less capable of phagocytic function than in adult life.

Certain authors (208, 209) have asserted that the age of the leucocyte itself as judged by nuclear configuration according to the Arneth or Schilling classifications may influence its capacity as a phagocyte. The majority of workers (210, 211, 212) who have made very accurate determinations, however, find no significant difference between the groups. We cannot, therefore, conclude that cellular maturity and phagocytic activity are in any way correlated.

Resistance of Bacteria to Phagocytosis. We have earlier in this chapter emphasized the necessity for employing bacteria of a known degree of virulence in phagocytic studies, since variations in this attribute lead to very wide divergencies in the readiness with which the organisms may be ingested. The close relationship between phagocytosis and virulence was first recognized by Denys and Marchand in connection with streptococcus immunity. Marchand (144) showed that leucocytes would take up non-virulent streptococci in the presence of normal serum, but that under similar conditions virulent streptococci were not phagocyted at all or only to a slight degree. He further determined that this resistance depended upon a condition of the bacterial body and not upon substances secreted and given off to the environment. Similarly Bordet

(213), studying the behavior of the same organisms in the peritoneal cavity of normal and immunized guinea pigs, noted that the leucocytes of the former failed to ingest the virulent encapsulated streptococci, which continued to multiply unchecked with the resulting death of the animal. Mennes (145) established similar facts in connection with the pneumococcus. These classical experiments were corroborated and extended by many others, such as those of Gruber and Futaki (214) and Preisz (215) with anthrax and Rosenow (172) with the pneumococcus.

With the recognition of the phenomenon of bacterial dissociation and its correlation with virulence, it was soon learned that in most instances the S or virulent form was resistant, the R form susceptible to phagocytosis in normal serum. This was found to hold good in the cases where the serum was derived from an animal belonging to a species susceptible to a given microorganism. This species relationship is admirably illustrated by the experiments of Robertson and Sia (75) on the bactericidal and phagocytic properties of normal sera from different species on the pneumococcus.

The explanation for this difference in the ease with which the virulent and avirulent variants are ingested by phagocytes depends upon differences in antigenic structures. The type-specific polysaccharides of the pneumococcus, for example, have been shown to combine with type-specific antibody and thus render it unavailable; otherwise it would unite with the same substance on the bacterial cell and so opsonize it. In the absence of the antibody, the polysaccharide situated at the surface of the bacterium manifestly possesses physico-chemical properties which render phagocytosis impossible. In the normal serum of susceptible animals, such as the rabbit, no type-specific antibody homologous for Pneumococcus Type I carbohydrate has been found. Therefore no phagocytosis in the normal serum and cells of this animal takes place when encapsulated organisms are added. This has been plainly demonstrated by the researches of Sia (74) and of Ward (68, 81). Similarly with other organisms a close relationship between antigenic structure, virulence, and phagocytosis has been revealed as a result of the many researches carried out in the recent past. As examples we may mention the work of Felix and Bhatnagar (216) on the differences in these respects between strains possessing the Vi or "virulent" antigen and those of less pathogenicity, and the experiments of Lyons and Ward (67, 217) on certain variants of the streptococci.

As we have also remarked in a previous section, another kind of variation in bacteria which is of the greatest importance in the study of the mechanism of phagocytosis is that which occurs during the process of growth. It has been known for some time that the organisms derived from young cultures may be more virulent as determined by the numbers

in the minimum lethal dose than those taken from older cultures (218). Moreover, it has also long been known but not sufficiently emphasized that such young organisms exhibit surface properties different from older ones and if the species is one which forms a capsule, this is frequently well developed during the early stages of cultivation, but may soon be lost upon further incubation (215). Working with the pneumococcus, Ward and Enders (68) and Shaffer and Enders (69) have shown that young (6–10 hours) cultures of this organism may be quite resistant to phagocytosis by the normal serum of man and the rabbit, whereas with the passage of time the organisms gradually undergo capsular damage and loss and become susceptible to phagocytic attack. The same phenomenon has been shown by Seastone (66) and Ward and Lyons (217) to take place in cultures of hemolytic streptococci as incubation is continued. We have some indication that under certain conditions the same loss of capsule and consequent ingestion may take place within the animal body following a preliminary multiplication with full capsular development (85).

In the light of these facts it becomes very apparent that both the dissociative phase and the growth phase of a pathogenic bacterium are essential factors in determining whether or not it will be taken up by phagocytic cells.

The Fate of Bacteria Ingested by Phagocytes. In the beginning of this chapter we have emphasized the fact that the mere engulfment of a living particle such as a bacterium by a phagocytic cell can by no means be taken as evidence that its death and intracellular digestion will ensue. Indeed many of the early students of the subject denied any direct participation of the cell in the destruction of the organism, believing that this was brought about by the lytic action of serum antibodies and complement. The swelling, granulation, and fragmentation of the ingested organism, which was frequently observed to take place following phagocytosis, was interpreted by them as a result of lysis which would have happened whether or not ingestion had occurred. In those instances in which no complement was present in the serum, it was held that the leucocyte furnished this factor. These views are no longer entertained, since it has become evident from the work of Neufeld (219), Hyde (220), and many others that leucocytes do not furnish complement, and that the lytic process is different from that of intracellular digestion.

The conception, however, that phagocytosis is not invariably followed by the death of the organism has been entirely upheld by subsequent investigation. Nevertheless, in many instances destruction of the bacteria does follow. Indeed this means of defense is probably the most important which the body possesses in disposing of invading organisms. The parallelism between phagocytosis and immunity in certain specific

infections was first revealed by the work of Metchnikoff and his school. In this section we will briefly review the evidence for correlation between the degree of phagocytosis and the resistance to infection and discuss the conditions under which phagocytic ingestion is followed by the death of the parasite.

In Metchnikoff's earliest work upon the daphnia or water-flea, he observed that when phagocytosis of the invading pathogenic yeasts was energetic, the daphnia recovered. When the yeast cells penetrated the intestinal wall of the daphnia in large numbers, and were enabled to multiply before the phagocytes could accumulate, then the body of the daphnia was soon overwhelmed with the parasites and the host died.

The original study upon the daphnia led to analogous experiments upon higher animals, and it soon became evident that in many infections there was a correlation between the resistance of the infected subject and the degree of phagocytosis.

Earlier studies concerned themselves chiefly with natural immunity. The infectious disease which at that time had been most thoroughly studied was anthrax, and Koch had shown that frogs and other cold-blooded animals were resistant. Taking advantage of this observation, Metchnikoff studied the phagocytosis of anthrax bacilli in frogs and found that it took place rapidly and effectively, all of the injected bacilli being soon engulfed by leucocytes. In the same way active phagocytosis of these organisms was demonstrated in such naturally resistant animals as dogs and chickens, while almost no ingestion occurred in susceptible animals like guinea pigs and rabbits. Rats — more resistant to anthrax than guinea pigs, less so than dogs — showed a degree of phagocytosis intermediate between that observed in the case of the other animals mentioned above. Paradoxically in these more susceptible animals, the normal bactericidal action of the blood serum upon anthrax bacilli, though never extreme, was often more marked than that of the naturally immune animals. Without entering into a discussion of this apparent anomaly between *in vivo* and *in vitro* results in respect to the bactericidal action of serum, it is evident that the experimental facts in this case strengthened Metchnikoff's conception of the significance of the leucocytic function in immunity against anthrax infection.

Metchnikoff's insistence upon the importance of the phagocyte in anthrax immunity has been in the main borne out by subsequent researches, although even today the purely humoral factors which may be involved are not clearly understood. As early as 1895, Marchoux (221) attributed the greatly increased resistance of actively and passively immunized rabbits to reinfection with virulent anthrax bacilli to phagocytic attack and subsequent destruction. As indication of intra-

cellular digestion, he noted alteration in staining properties, granulation of the rods, and their final disappearance. Later Preisz (215) in an extensive investigation, found that the phagocytes, when not impeded by the development of capsules, were the chief agents in the destruction of these bacilli.

As we have noted in the previous section, studies analogous to those originally made with anthrax by Metchnikoff were subsequently carried out by Bordet, Marchand, Mennes, and others with the streptococcus and pneumococcus in which results coinciding with his were obtained. In each case resistant animals showed marked phagocytosis, whereas susceptible ones failed to exhibit it to the same degree. The observations which have been cited above concerning the inverse relationship which exists between the virulence of the pathogen and its susceptibility to phagocytosis tend strongly to uphold the conclusion that host resistance in many cases depends upon the phagocytosis and consequent destruction of invading organisms.

Further support for this point of view cannot be considered with anything like completeness, since the pertinent literature is very extensive. We may refer briefly, however, to certain of the more recently reported observations.

In 1916, Keyes (222) showed that pneumococci after introduction into the blood of pigeons were rapidly phagocyted and destroyed by the fixed tissue cells of spleen and liver. On the whole he found little evidence for the participation of the circulating leucocytes in this process. Furthermore he showed that the serum of these birds did not possess any bactericidal action upon the pneumococcus — a fact which strengthens the conception of the essential participation of the cells in killing these bacteria.

Similarly, in 1926, Hedley Wright (83) demonstrated the predominant role of the phagocytes, particularly those of the reticulo-endothelial system, in the resistance of rabbits to infection with Pneumococcus Type I. When this organism in its avirulent form is inoculated intravenously into normal rabbits, it is rapidly and permanently eliminated from the blood. This is mediated largely by phagocytic cells of the lung, liver, spleen, lymph glands, and bone marrow. Following intravenous inoculation, virulent organisms, on the other hand, are temporarily reduced in numbers or completely removed, but within a few hours reappear in the circulating blood, in increasing amounts until the animal succumbs. In the actively or passively immunized animal, virulent organisms behave in a manner identical with the avirulent variety in the normal rabbit. They are opsonized by the available antibody and are rapidly removed from the blood by the phagocytic cells. Ørskov (223) and his associates in Madsen's laboratory have carried out

a large series of experiments with the object of determining the exact pathogenesis of a variety of peroral infections. In the greater part of this work they have utilized paratyphoid bacilli. In mice, these organisms, after traversing the intestinal mucosa, enter the lymphatic channels, whereby they are carried to the regional lymph glands and eventually reach the blood stream, whence they are removed by the cells of reticulo-endothelium. Cultivation experiments showed that destruction may occur in such organs as liver and spleen but that certain of the bacteria persist in these sites, particularly in the lymph glands, where they may increase and thence generally reinvade the body. Cannon and his co-workers (84, 224), in somewhat the same type of experimentation, have employed various organisms, including the staphylococcus, paratyphoid bacillus, and pneumococcus. The phagocytic cells in these cases were found to be responsible for the removal of such bacteria from the blood stream of rabbits, and evidence obtained by cultivation of weighed and ground tissues indicated that considerable destruction followed upon their attaining an intracellular position. The efficiency of this process is greatly increased in specifically immunized animals.

Enders and his associates (85) investigated Pneumococcus Type III and used rabbits as the experimental animal. They found that disappearance from the blood stream was conditioned by the increasing impairment of the capsule as cultivation was continued. Under these circumstances the fixed tissue phagocytes not only took up but killed large numbers of virulent organisms introduced into the circulating blood. Their destruction was found to be comparatively rapid, since it was marked within one to two hours following the introduction of the organisms into the body.

In their studies on local immunity Gay and his co-workers (225, 226, 227, 228) have shown that the accumulation of macrophages in the pleural cavity definitely increases the immunity of rabbits to hemolytic streptococci inoculated into that area. The effectiveness of these cells is due to their phagocytic activity against this organism. Nakahara reported a similar increase in the resistance of normal mice to intraperitoneal pneumococcus infection, following the mobilization of macrophages in the peritoneum. These results cannot be accepted unreservedly, however, since Clark (88), working with rabbits, failed to obtain protection against pneumococcus by merely producing a macrophagic exudate within the pleural cavity, although she did secure excellent results under these same conditions when antiserum was likewise employed. Goodner and Miller (229) have reported that there is a correlation between the number of monocytes present at the time of infection in the peritoneal cavity of mice and their resistance to Pneumococcus Type I mixed with a suitable quantity of antiserum. Those animals

having only a few of these cells initially present succumb following the same intraperitoneal dose of antiserum and organisms which is tolerated by others exhibiting a greater number of monocytic cells.

In an attempt to analyze the immunological factors in recovery from lobar pneumonia, Robertson and his collaborators (230, 231) have correlated the appearance of macrophages with the decrease or disappearance of pneumococci in local areas of consolidation in the lungs of dogs. Although recovery does not inevitably follow macrophagic assemblage, the data secured by these authors indicate that in those cases where there is a failure of these cells to respond the animal invariably dies. Moreover, they have noted very definite phagocytosis and signs of intracellular destruction of the ingested organisms in the macrophages in the pneumonic lung. The findings of Nye and Harris (232) do not entirely agree with these results, since they have noted that the pneumococci become greatly reduced in numbers in consolidated portions of the lung before the macrophagic reaction occurs. They have observed evidence of active phagocytosis by the polymorphonuclear leucocytes at about the time the organisms are decreasing, but are by no means convinced that their disappearance is mediated solely by these cells.

The important role played by clasmatocytes in clearing various local sites and the blood stream of organisms has been emphasized in the earlier studies of Buxton and Torrey (233), Dudgeon and Ross (234), Beattie (235), and later by Jelin (236).

In this incomplete summary of experimental results which indicates that in the animal body the phagocytosis of pathogenic bacteria is frequently followed by their death, the role of the reticulo-endothelial cells in performing this function has been particularly stressed. The polymorphonuclear elements are none the less of great importance, not only in clearing the blood stream of invaders, but also in dealing with organisms localized in a given area. These cells are the first to appear in sites of inflammations, and, when the surface properties of the organisms are suitable, rapidly engulf large numbers of parasites. From the early observations of Metchnikoff, Bordet, Denys, Marchoux, and many others, as well as the experimental work *in vitro* on the bactericidal power of polymorphonuclear leucocytes which will be reviewed below, there can be no question in respect to the capacity of these cells to effect the destruction of large numbers of bacteria under suitable conditions. In the body of susceptible animals, however, they, like the clasmatocytes and fixed tissue phagocytes, may fail or at least seem to fail to destroy the organisms which they have ingested. Thus, it has long been held that phagocytosis of tubercle bacilli, which takes place quite regularly following the introduction of these organisms into the body, does not result in their destruction. On the contrary, it is asserted that the leucocyte forms

a safe nidus where pullulation may occur, as well as a vehicle by which a transport to distant tissue sites may be accomplished. It is also most probable that in localized lesions due to staphylococci these organisms remain viable, although easily ingested by the enormous numbers of neutrophiles which have accumulated. Similarly, macrophagic phagocytosis of the staphylococcus in susceptible animals, such as the rabbit, although it readily takes place, is often insufficient to completely sterilize the blood and prevent a fatal termination, as the work of various authors, the most recent of whom is Lyons (237), clearly shows. Many observers, struck by the clear-cut outlines and sharp staining properties of phagocyted gonococci in pus and spinal fluids, have felt that these organisms were viable. However this may be, it is certain that the activity of the polymorphonuclears in these cases, although usually vigorous, does not immediately, at least, lead to the rapid elimination of the cocci. An additional instance of pronounced phagocytosis with little or no indication of intracellular death is to be found in the ingestion of leprosy bacilli by large mononuclear cells.

Numerous experiments carried out in the test tube testify to the killing of bacteria by leucocytes. This fact becomes most evident when we consider various pathogenic organisms, notably the Gram-positive cocci, which are, as we shall see, practically completely resistant to the action of either normal or specific immune serum alone whether or not complement be present. There are nevertheless a few observations which suggest that serum constituents alone, without the assistance of phagocytic cells, may exert a feeble bactericidal effect. Mackie and Finkelstein (238) reported that the normal heated sera of a variety of animal species killed or inhibited the growth of a small number of pneumococci and streptococci. Wulff (239) and, more recently, Tillett (240) have described bactericidal properties against streptococci and other pathogenic bacteria resident in the sera of patients with pyrexias of varied origin. These disappear with the cessation of fever and convalescence. The significance of this phenomenon is not manifest at present, but it is probable that no specific antibody mechanism is involved.

Although we cannot entirely disregard such observations, the results of most investigators are entirely unequivocal in showing that specific antibody, whether in normal or immune sera, has no inhibiting or lethal action upon the growth of either pneumococci or streptococci. That pneumococci grow well in homologous antiserum is a matter of common observation. Even when complement is added, we have never seen any indication in such systems of inhibitory power against the pneumococcus. Robertson and Sia (60) likewise were unable to detect pneumococcidal properties in the fresh sera of naturally immune animals such as the

cat, dog, and pig Heist and the Solis-Cohens (241) in earlier work had also found that the serum of the resistant pigeon failed to kill pneumococci.

It has likewise long been known that the serum of various animals is of itself incapable of killing streptococci. Thus, in 1897, Denys and Marchand (242) and Bordet (213) in their classical studies of streptococcal immunity stated that neither normal nor specific immune serum is bactericidal for these organisms. Since in the case of the staphylococcus it is difficult in test tube experiments to demonstrate any destruction or inhibition even in mixtures of serum and leucocytes, it is evident that the former alone has no effect.

From such considerations it is apparent that the killing of streptococci, pneumococci, and without much doubt some other Gram-positive organisms, requires an additional factor or factors. It has been repeatedly demonstrated that an essential constituent in this type of bactericidal mechanism is the phagocyte. This was shown many years ago by the experiments of Denys and Le Clef (53). In mixtures of polymorphonuclear leucocytes and fresh normal rabbit serum growth of streptococci was unrestrained. Upon the addition of specific antiserum to such mixtures a marked inhibition in their multiplication occurred. In drawing conclusions from this observation one must bear in mind that both sorts of serum alone are without effect. Bordet (213) was unable to obtain the same result, but in the light of the modern work of Todd (78), Hare (79, 80), and others, who have demonstrated the streptococcidal power of leucocyte-serum mixtures, there can be little doubt of the accuracy of Denys's findings. Analogous experiments by Robertson and his associates (60), employing a mixing device similar to that described by Todd, have demonstrated that mixtures of fresh normal sera of various species of animals and polymorphonuclear leucocytes exhibit the capacity to destroy many thousands of virulent pneumococci. Since the serum alone is innocuous, it is necessary to conclude that killing only follows phagocytic ingestion which can be easily observed to have taken place. The results are entirely comparable when type-specific immune serum is substituted for normal serum, provided complement be present, as the investigations of Ward (81) have demonstrated. In all of these studies we have mentioned the vast majority of cells were polymorphonuclears. In so far as we are aware, the bactericidal properties of mononuclear cells, *in vitro*, have not been examined by means of the same techniques. We do not doubt that this kind of cell would reveal equal, if not superior powers in the destruction of virulent bacteria.

Although the data to which we have alluded unequivocally show that the phagocyte can kill the organism which it has ingested, they by no means suggest that the serum constituents — antibody and complement,

— play no part in the process of destruction. On the contrary, there is ground for believing that both in certain cases may be necessary, although final proof is lacking. It will be remembered that heated immune serum can induce a marked degree of phagocytosis. In spite of this, destruction of virulent pneumococci does not usually occur in mixtures of such serum and leucocytes. One of the writers with Ward in unpublished experiments carried out numerous experiments in which mixtures of washed leucocytes, normal heated serum, and heated antiserum were tested for their capacity to kill virulent pneumococci. In all but one instance, as few as from one to ten organisms would survive and multiply in these mixtures, although the leucocytes were capable of ingesting large numbers of cocci. But when they employed normal heated serum and R pneumococci an unmistakable bactericidal effect was secured. Robertson and Sia (75), working with normal pig serum, heated to 56° C., and leucocytes, observed no pneumococcidal effect in spite of the fact that a considerable degree of phagocytic action could be demonstrated, whereas the same serum unheated and added to the leucocytes induced excellent killing. Conversely, the cells by themselves or extracts obtained from them did not injure the virulent pneumococci.

Two explanations of these results are possible. Either a few organisms escape phagocytic ingestion in the absence of complement, or intracellular digestion cannot usually take place without it. In view of the marked activity of the leucocytes, in our own experiments we are inclined to consider the latter hypothesis as more probable. But until intracellular multiplication of organisms or their digestion is observed microscopically in the absence of complement, no final choice between these alternatives can be made.

We must also leave open the question concerning the role of antibody in the intracellular mechanism of destruction. From experiments carried out in the test tube, it would appear reasonably well established that under certain conditions ingested organisms may survive within leucocytes. Rous and Jones (243), in 1916, showed that phagocytized typhoid bacilli were protected from the action of lytic antibody and potassium cyanide and survived in the cells. Moreover, they found that erythrocytes which had been taken up by polymorphonuclears did not undergo hemolysis when a specific hemolytic antiserum was added to the mixture. From experiments in which they repeatedly washed the cells derived from the spinal fluids of cases of *H. influenzae* meningitis and then inoculated onto agar plates, the leucocytes thus freed from the vast majority of extracellular organisms, but which still contained numerous phagocytized bacilli, Fothergill and his associates (244) secured evidence which strongly suggests that the intracellular influenza bacilli remain viable and capable of active proliferation. The recent

work on staphylococcal antibacterial immunity of Lyons (237) makes it quite clear that toxigenic strains, although they are easily ingested in normal human blood, having been opsonized by factors in the serum, are not killed by the leucocytes. In normal rabbits the phagocytic mechanisms fail to sterilize the blood completely. But in the presence of an immune antibody against the capsular substance of the staphylococcus, permanent sterility of the blood frequently follows intravenous inoculation. This suggests that the phagocytosis of the organisms thus opsonized terminates in their extinction.

Before we are able, however, to write a clear description of the function of antibodies in the intracellular digestion of bacteria, much additional investigation is required. At the moment the meager data suggest that along with complement some sort of antibody may be on occasion indispensable factors in the process.

This conception of the participation of complement and antibody in the intracellular dissolution of certain microbes which has been suggested by recent analyses of that bactericidal mechanism demanding the presence of leucocytes is somewhat analogous to the original belief of Metchnikoff, who, however, erroneously assumed that the destruction of all bacteria depended either directly or indirectly upon the phagocytic cells.

Metchnikoff (1, 245, 246, 247), basing his opinion on his own studies and those of his pupils, came to the conclusion that normal blood contains no free bactericidal substances for any species or organism. He assumed that these substances were constituents of the various phagocytic elements, by means of which the cells digest the foreign materials which they take up. He believed that there were two varieties of such digestive enzymes or "cytases" just as there are two varieties of phagocytes. The microphages formed the "microcytase" which he identified with alexin or complement concerned in the bactericidal effect of serum, whereas the "macrocytase" was a product of the large mononuclear, lymph, and endothelial cells concerned in the lysis and digestion of red blood cells and other non-bacterial materials. In normal animals the destruction of invading bacteria or of injected cellular substances was held to be accomplished entirely by the phagocytic process with subsequent digestion by the cytases alone. In immunized animals, however, there is present in the circulating blood another substance, not identical with the cytases, but also considered by Metchnikoff to be derived from the leucocytes or blood-forming organs — the "fixateur" (Ehrlich's "amboceptor," Bordet's "sensitizer"). Metchnikoff maintained that this specific antibody prepared the bacteria in some manner for the digestive action of the cytases. Since normally the latter existed only in the cells, cleavage of sensitized antigens could take place only intra-

cellularly unless by previous leucocytic injury these enzymes had been liberated into the plasma.

It is self-evident that the unqualified acceptance of this interpretation is dependent upon the demonstration that the circulating blood normally contains no alexin or complement. Although this was maintained by Gengou (248) on the basis of experiments involving the titration of complement in plasma, in contrast to serum removed from the clot, whereby the leucocytes are injured, the majority of subsequent investigators have failed to confirm Gengou's observations, and agree that alexin exists in the fluid portion of the blood as such in the animal body. Also contrary to Metchnikoff's conception is the demonstration by Zinsser (249), Hyde (220), Neufeld (219), and others that alexin is not a product of the leucocytes.

Thus, although Metchnikoff's theory as a whole cannot be accepted today, there is a possibility that he was correct in postulating an essential role for both immune bodies and complement in the intracellular destruction of at least certain microorganisms. Nevertheless, since these two elements by themselves or in conjunction cannot bring about microbial death except in the case of those organisms which are susceptible to extracellular lysis, the living cell must furnish additional factors which are essential to the process. The nature of these is not known, but certain substances having themselves bactericidal effects, although usually only to a moderate degree, as well as a variety of enzymes, have been obtained in leucocytic extracts. It is possible, though not proven, that some of these may be concerned in the mechanism of intracellular digestion.

Bactericidal Substances and Enzymes of Leucocytic Origin. Denys and Kaisin (250) in 1893 found that the pleural exudate of rabbits, obtained by injection of dead staphylococci and freed of cells by centrifugation, was bactericidal for living organisms of the same species. At about the same time, Buchner (251) noted similar action of exudative fluids obtained with aleuronat on *E. coli*. Analogous results were obtained by Hahn (252) with *B. typhosus*. Denys and LeClef (53) in their fundamental researches on the streptococcus reported a marked inhibitory action of the cell-free exudate on this organism. Their observation was confirmed by Bordet (213), who found that when the fluid was heated for a few minutes at 60° C., it lost its activity. He was unable, however, to correlate the *in vitro* results with a similar inhibitory effect of such extracts in streptococci developing within the animal body. Some of these workers, such as Buchner and Hahn, had regarded these bactericidal substances as identical with alexin. Schattenfroh (253, 254) showed that since they were somewhat more thermostable, this view was probably erroneous. Subsequent study by Moxter (255),

Schneider (256), Zinsser (249), Petterson (258, 259), and Kling (257) have confirmed Schattenfroh's conclusions. Petterson, moreover, showed that these substances are more active toward certain species of bacteria than toward others. Schneider termed these endocellular bactericidal substances "leukins," by which name they are still known today.

Experiments upon the protective and therapeutic action of leucocytic extracts followed logically upon the discovery of antibacterial properties in leucocytic extracts. Bordet's observations already cited are among the earliest attempts in this direction. Petterson (260) studied the problem more fully, and came to the conclusion that such reagents had a favorable effect in anthrax infections which, however, he admits was not very great. Hiss (261), following the same line of investigation, at first worked with extracts in distilled water of dog, rabbit, and guinea pig leucocytes. Later he confined himself entirely to rabbit cells. Both clear supernatants and cellular residues were usually injected. With this material, Hiss treated rabbits infected with a variety of pathogens such as *E. typhi*, pneumococcus, streptococcus, and meningococcus, and obtained results which indicated that in all these cases the leucocytic extracts exerted a protective action. Subsequently, Hiss and Zinsser (262) treated series of patients ill with meningitis, pneumonia, and staphylococcus infections with extracts prepared by the method of Hiss, and concluded that in many cases the course of the disease was thereby favorably influenced. The mechanism whereby such infections are influenced remains obscure, since Hiss and Zinsser were able to show that the extracts did not favor phagocytosis and that their moderate bactericidal activity could not account for their effectiveness. At present, we are inclined to believe that the beneficial effects are to be attributed to those obscure factors which account for the not infrequent successes of so-called non-specific protein therapy, which consists in the injection of almost any bacterial or other protein.

Leucocytic Enzymes. In addition to bactericidal substances extracted from leucocytes, a number of true enzymes have been obtained. Among the earliest observations in this respect are those of Hammarsten (263), who showed that pus cells digested fibrin, and of Leber (11), who noticed that sterile pus could liquefy gelatin. It has long been remarked in sections of staphylococcus abscesses that a ring of digested or degenerating tissue is formed about an accumulation of leucocytes. Such leukoproteases have been studied in detail by Opie (264, 265, 266, 267), Barker (268, 269), Müller and Jochmann (270, 271). Although the two last-mentioned authors denied the existence of protease in monocytic cells, Opie found that two distinct proteolytic enzymes could be extracted from the cells of exudates obtained by injection of turpentine. One —

peculiar to the polymorphonuclear leucocyte, and similar to that previously described by F. Müller (272) — acts in a weakly alkaline reaction. The other — derived from exudates composed largely of monocytic cells — acts in a weakly acid reaction. Jochmann (273) states that the neutrophilic leukoprotease acting upon casein produces tyrosine, tryptophane, and ammonia. He concludes that functionally it is like trypsin. The proteases of the polynuclears appear to be found only in the cells of certain animal species and only in those which possess true neutrophilic granules. Thus, according to Müller (274) and Tschernorutzki (275), they are found in the granulocytes of man, catarrhin apes, dogs, cats, and panthers, while they are absent in the corresponding cells of rabbits and other rodents as well as horses, platyrrhin apes, and birds.

The question arises: Are these leukoproteases identical with the bactericidal substances extracted from leucocytes as described above? For it might be that bacterial death resulted merely from the digestive action of the enzymes. Jochmann (276) answered this in the negative. By repeated alcohol precipitation of glycerin extracts of leucocytes, he obtained an enzyme which possessed no bactericidal properties, though it still was actively proteolytic. The leukoproteases, therefore, are unable directly to destroy living bacteria, and accordingly their significance in immunity is not clear. The possibility however remains that they may take part in the killing of bacteria provided other factors are present. But their principal function seems to consist in the resorption of dead tissues, fibrin, blood clots, etc. E. Müller (274) pointed out their possible importance in the rapid removal of the massive fibrinous exudates remaining after the crisis in lobar pneumonia.

Lipases are also found in the white blood cells. Poulain (277) was the first to demonstrate clearly the presence of this sort of ferment in these cells. Bergel (278, 279) and others have regarded them as the characteristic ferments of the monocytic series exclusively, while others have claimed to have demonstrated their presence in granulocytes. Bergel's view is that most currently held today. The lipolytic properties of macrophages may be of significance in respect to immunity in tuberculosis and leprosy, since there is general agreement that when the causative organisms of these diseases are destroyed, this takes place in these cells rather than in the neutrophiles.

A number of other ferments have been demonstrated in leucocytes. These have been described, and much of the pertinent literature has been summarized by Fleischmann (135). The activity of enzymes capable of splitting various complex and simple carbohydrates such as starch, glucose, mannose, levulose, and lactose has been revealed as well as that of others acting upon nucleic acid, hydrogen peroxide, and some of the purine bases. Of considerable interest is the recent discovery by

Dubos (280) of an enzyme in polymorphonuclears which alters the Gram-staining properties of pneumococci. It is possibly a nuclease.

In this chapter we have attempted to give a sketch of the various biological elements which participate in one of the two fundamental mechanisms of immunity. These comprise the different varieties of cells which possess the capacity to ingest bacteria, the physical and chemical conditions, in so far as they are known, which favor or inhibit this tendency of the phagocyte, and the extremely significant functions of normal and immune bodies and of alexin.

The manner in which these factors — some of them most complex, and therefore capable of an altered behavior under slightly changing conditions — interact to attain the elimination of injurious agents from the body, has become somewhat clearer with the recording of experimental data as time has passed. But we are still far from possessing a completed picture. We remain almost entirely ignorant of the vitally significant details of the process by which the cell overcomes the parasite once it has been ingested. Careful studies by means of the techniques for single cell isolation may assist in the analysis of this mechanism. We require more exact information concerning the relative bactericidal powers in normal and immune sera of polymorphonuclear leucocytes and macrophages, which can possibly be obtained by careful analyses carried out in the test tube. Although a few attempts, not mentioned in the text, have been made to study the behavior of leucocytes from the hypersensitive organism following their ingestion of the specific antigen, we believe that further researches on this problem could be profitably carried out. The ancient question in regard to whether or not complement alone is capable of inducing phagocytosis, at least in so far as concerns non-pathogenic bacteria and inert substances, has not been finally answered.

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CHAPTER XII

HYPERSENSITIVENESS (GENERAL PRINCIPLES)

Hypersensitiveness is an individual increased specific reaction-capacity to a substance which, in normal subjects of the same species, produces little or no reaction. In its immunological sense, the term "hypersensitiveness" implies that the increased susceptibility is the result of a reaction between the responsible substance and the cells, mediated by a specific mechanism not present, or present to an ineffective degree, in the normal individual. In protein anaphylaxis, which represents one of the important classes of hypersensitiveness, antibodies, or antibody-like substances are known to be involved. In some other forms an antibody-like mechanism has not yet been demonstrated. But the reactions are in all forms analogous in so many ways that similar mechanisms are probable. Observed differences depend very largely upon the physical (molecular weights, sizes, etc.) and chemical properties of the responsible incitants.

The subject of hypersensitiveness is one that has become an important chapter of immunology and has revealed more delicately than any other branch of the subject the specific changes of reaction-capacity which the animal body undergoes in contact with foreign antigenic materials.

Early Observations. The earliest observations regarding hypersensitiveness were made in the course of experimentation with diphtheria and tetanus toxins. These antigenic substances were toxic in themselves, a property which somewhat confused the basic principles involved in the phenomenon. We will for the present, therefore, limit our discussion to those forms of hypersensitiveness which are incited in animals and man by various protein antigens which possess little or no toxicity in themselves. This type of hypersensitiveness is generally referred to as protein anaphylaxis, or simply as anaphylaxis.

The earliest observation having direct bearing upon anaphylaxis is one which Morgenroth discovered in the writings of Magendie. Morgenroth (1) mentions that, in his "Vorlesungen über das Blut," published in 1839, Magendie describes the sudden death of dogs which had been repeatedly injected with egg albumen.

There can be little doubt as to the anaphylactic nature of Magendie's results.

A clear statement of the phenomenon was given, also, by Flexner (2), in 1894. In describing certain experiments he says: "Animals that had withstood one dose of dog serum would succumb to a second dose given after the lapse of some days or weeks, even when this dose was sublethal for a control animal."

However, the isolated observations recorded above were neither correlated nor followed out to their logical developments, and a systematic study of the problem was deferred until Richet and Portier (3) attacked it in 1902.

Richet and Héricourt (4) had observed in 1898 that dogs treated with eel serum, which is toxic *per se*, could be killed by a second injection of an amount too small to injure normal untreated animals. Some years later Richet, in collaboration with Portier (3), determined a similar fact in the case of "actinocongestin," which they isolated from the tentacles of actinia.

Richet and Portier's observations were as follows: Actinocongestin injected intravenously into dogs in quantities of 0.05 to 0.075 gram per kilo weight may cause illness, with vomiting, diarrhea, and respiratory distress, but does not kill. A dose of 0.002 gram per kilo causes no symptoms in a normal dog. If, however, 0.002 gram of the poison is injected into a dog which has previously received a sublethal dose and recovered, the result is violent illness and often death.

They therefore spoke of the phenomenon as "anaphylaxis" ("action anaphylactique de certains venins"), to express its antithesis to prophylaxis or protective effects.

The attention of investigators was again focused upon the problem by the publication of Arthus (5) in 1903 on the repeated injection of horse serum into rabbits, and by observations made upon guinea pigs by Theobald Smith in 1904.

Arthus (6) found that horse serum injected into rabbits by any of the usual paths is entirely innocuous. It is possible to inject 10, 20, or even 40 cc. without harm. If, however, one repeatedly injects small amounts, 5 cc. or less, subcutaneously, at intervals of several days, eventually the later injections will give rise to infiltrations, edema, sterile abscesses, and even gangrene at the points of injection. He recognized that this was not due to cumulative action, and that it was not necessary to inject in the same place to produce the characteristic response. For instance, the early injections might be made into the peritoneum, the subsequent ones into the skin. He recognized the phenomenon as analogous to the observations of Richet.

The "phenomenon of Theobald Smith" was observed in the course of the standardization of diphtheria antitoxin. It was noticed that guinea pigs which had been used for this purpose and had survived had acquired susceptibility to subsequent injections of normal horse serum made several days or weeks later.

At the same time that these observations on animals were being made, von Pirquet and Schick were studying the peculiar phenomenon known as Serum Disease which occurred in human beings usually from 7 to 12 days after antitoxin injections. Their book, "Die Serumkrankheit," appeared in 1906, and it is important to note that the conclusions which they reached concerning the essential mechanism of Serum Disease were borne out subsequently in every detail by later studies in animals and man. The first systematic animal experiments on anaphylaxis which established the

fundamental facts were those of R. Otto of the Frankfurt Institute of Experimental Therapy and those of Rosenau and Anderson carried out at the Hygienic Laboratory of the United States Public Health Service.

Although the paper of Otto (7, 8) appeared in print a little earlier than did the first one of the American workers, the investigations were independent and almost synchronous. Their results, moreover, confirm each other in all essentials. Otto showed that the Theobald Smith phenomenon was independent of the toxin or antitoxin contents of the injected serum, and could be produced with horse serum alone. He also showed that, while a preliminary injection of horse serum "sensitized" a guinea pig to a subsequent dose given after an interval of 10 to 12 days, the repeated injection of considerable quantities at short intervals produced a condition of "antianaphylaxis" or immunity to the later injections.

In the chapter following we shall discuss the theories of anaphylaxis at considerable length. Our present purpose is to present the experimental facts of protein anaphylaxis. Details of the mechanism, the site of the reaction, its physiology in various animals, and our knowledge concerning varieties of hypersensitiveness will be more easily comprehended when the basic facts of protein sensitization have been discussed.

The Antigen. The substances which incite anaphylaxis are antigens and need not be separately dealt with in this connection. Everything that we have said about antigens in general in the chapter devoted to the subject is applicable to the substances which sensitize animals, the sensitizing property being merely another expression of their capacity to form antibodies.

The experimental results of Rosenau and Anderson (9), who observed toxic effects on reinjection of sensitized guinea pigs with digested horse serum, may have been due to remaining traces of coagulable protein, since Wells found that 16 months' digestion with trypsin did not completely destroy the coagulable material in beef serum.

In regard to altered antigens, in the sense of Obermeyer and Pick, Landsteiner (10), and others, the same specificity relations prevail as those discussed in the chapter on antigens.

Partial antigens or "haptenes" may induce shock, but will not sensitize — a fact of great importance in bacterial anaphylaxis. The problems arising from this fact in connection with many forms of human hypersensitiveness have been much clarified by studies of compound antigens. This matter will be further discussed below.

Specificity. *The anaphylactic reaction is specific,* within the limitations of specificity for immune reactions in general.

Animals and man can be rendered separately sensitive at one and the same time to a variety of antigenic substances, reacting specifically to each on second injection.

The relationship of chemical structure to specific reaction has been

demonstrated repeatedly by anaphylactic reaction by Wells and Osborn.

As regards organ specificity, anaphylactic experiments have corroborated the facts revealed by other immunological methods, and have supplied a technique for such investigations far more delicate than either precipitation or complement fixation.

Kraus, Doerr, and Sohma (11) were able to show that animals sensitized with protein from the crystalline lens became hypersusceptible to lens protein generally, whether this came from the species from which the original lens was taken, or whether some other variety of animal had furnished it. On the other hand, animals so sensitized, while hypersusceptible to lens protein, did not react to injections of homologous blood (12). In other words, the lens contains a characteristic variety of antigen peculiar to this kind of organ throughout different animal species, but not common to other tissues and organs of the same animal. Results similar to these were obtained by von Dungern and Hirschfeld (13) with testicular protein, although here the phenomenon seemed to be less rigidly organ-specific than in the preceding case. These writers worked with the localized reaction described above as the *phenomenon of Arthus.* They injected extracts of the testicular materials into the ears of rabbits and incidentally made the observation that pregnant females would not infrequently react to a first injection without previous sensitization.

The possibility of "autosensitization" has given much opportunity for clinical speculation. Rosenau and Anderson (14) found that guinea pigs can be sensitized by means of extracts of guinea pig placenta. They have applied this to the possible explanation of eclampsia, and similar reasoning, as we shall see, has been utilized in many other conditions. Attempts have also been made to show, by the anaphylactic reaction, that the tissue of malignant tumors possesses such "tissue-specific" or "organ-specific" qualities. Yamanouchi (15) claims to have shown this, but his results were not confirmed by Apolant (16).

In regard to the "organ-specific" properties of such organs as the liver, spleen, kidney, blood, etc., Pfeiffer (17) has published results with the anaphylactic method which would seem to encourage the belief of the existence of such specificity. However, Ranzi (18) had previously obtained entirely negative results, and Pearce, Karsner, and Eisenbrey (19) failed to confirm Pfeiffer's claims.

By a similar process of reasoning Elschnig (20, 21, 22) has attempted to explain sympathetic ophthalmia. The destruction and absorption of injured uveal tissue, according to him, induce the formation of organ-specific antibodies by which the remaining uveal structures of the same, as well as of the opposite, eye are sensitized. The consequence is a "sympathetic" inflammation which "is to be regarded purely as an anaphylactic reaction."

The failure of many investigators to isolate filterable virus agents from various types of encephalomyelitis has led to much speculation regarding the possible autocytotoxic origin of such disease. That organ-specific sensitization might play a role in some of these conditions has likewise been suggested by the fact that about one of every 4000 people treated with 14 to 21 injections of rabbit brain material, in rabies prophylaxis, develops some form of paralysis which almost always clears up and is therefore very unlikely to be caused by rabies virus as such. Many observers have

attempted to produce injury of the central nervous system by injecting suspensions of homologous brain tissue, but no one obtained results of any consequence until Schwentker and Rivers (23) in 1934 produced paralysis in rabbits by the injection of brain material which had been subjected to slight autolysis. Later, the same writers (24) produced pathological changes, accompanied by myelin destruction, in the brains of 7 out of 8 macacus rhesus monkeys by repeated intramuscular injection of aqueous emulsions and of alcohol-ether extracts of sterile, normal rabbit brains. The number of injections necessary was very large, ranging from 46 to 85. These experiments done under careful control seem to indicate that a certain amount of organ specificity can be demonstrated for brain tissue under conditions of intense immunization. Whether or not such a process can be taken to explain cerebral lesions in actual disease is of course still questionable.

Active Sensitization. Active sensitization is a term analogous to active immunization in that it signifies that the animal develops its sensitiveness in physiological response to injection of the antigen. In contrast, in *passive sensitization*, the animal is rendered sensitive by virtue of antibodies transferred to it from the actively sensitized one.

Active sensitization, then, consists in administration of the antigenic substance to the animal.

As far as the *manner of administration* is concerned, the conditions are again analogous to those prevailing in immunization. Any manner of administration by which the antigen may come in contact with the tissue cells of the body without previous alteration by digestive ferments may lead to sensitization. This is synonymous with saying that any manner of introduction by which the antigen may lead to antibody formation may sensitize.

Spontaneous sensitization through the placenta, the skin, intestinal canal, and the respiratory passages has now been definitely determined, and the unchanged passage of foreign proteins through the mucous membranes of these regions plays a role of great importance in the spontaneous sensitization of man. This matter will be more extensively discussed in that connection where references are given to the experimental work carried out in animals.

The *quantities of antigen* which are necessary to sensitize vary considerably. All that is necessary is to introduce an amount of antigen sufficient to initiate antibody production. This will vary within very wide limits for the several antigenic substances. When working with substances as antigenically potent as horse serum, Rosenau and Anderson (14, 25) obtained sensitization after the injection of as little as one millionth of a cubic centimeter. Quantities almost as low as those reported by Rosenau and Anderson for guinea pig sensitization have been successfully used by Doerr and Russ (26), and still lower quantities of crystallized egg white have sensitized in the hands of Wells.

Moderate amounts are more effective in sensitizing for laboratory experimentation than extremely small quantities or very large ones. Wells (27) has found this to be the case, and it is not unlikely that the relative ineffectiveness of amounts which are too large may be due to a persistence of antigen in the circulation after antibodies have begun to form. As a consequence of this there may be a certain amount of protracted desensitization similar to that which occurs in serum sickness in man.

Incubation Time. The hypersensitive state develops at varying periods after the sensitizing injection. Though often so stated, it is not likely that the route of injection matters very much for, even though intravenous or intracardial injection means that the antigen comes into almost immediate contact with the tissues, subcutaneously injected antigen probably does not lag much more than 72 hours behind, and some of the antigen will probably get into the circulation immediately. The quantity, however, seems to influence the length of the incubation time considerably. In general, we can say that, while very large doses of antigen may increase the length of the incubation time, perhaps because of the prolonged presence of antigen in the circulation and the partial neutralization by this residue of the first-formed antibodies, no such prolongation results from moderate doses. Below a certain limit, the smaller the dose, the longer the incubation time, because of the slower development of antibodies. Repeated injection of guinea pigs with the same antigen during the period of incubation prolongs the incubation time because of the partial desensitization which follows each injection. Guinea pigs actively sensitized with 0.01 cc. to 0.1 cc. of a serum antigen become hypersensitive within 5 to 7 days, and generally exhibit their maximum sensitivity between the 10th and 20th days after injection.

Reinjection for the Production of Anaphylactic Shock. The best method of obtaining severe symptoms is by intravenous injection, since, in this method, the injected antigen comes into contact with the sensitized tissues suddenly. In animals that are not particularly sensitive to anaphylactic reactions, rabbits, dogs, etc., the intravenous method is the only one with which acute death can be obtained with any degree of regularity. When the injections are made subcutaneously or intraperitoneally, considerably larger amounts must be given in order to obtain the same results, since the speed of absorption of a reactive amount is an important factor in acute shock. Subcutaneous injection into sensitized rabbits produces local reactions consisting of edema and sometimes necrosis, a phenomenon spoken of as the *Arthus phenomenon.* This does not occur in the same way in guinea pigs and some other animals. It is quite common in man.

The severity of reaction can be diminished by gradual administration

of the antigen, either by injecting in a manner which leads to a slow absorption, by dilution, or by the repeated injection of fractions at short intervals.

The relationship between sensitizing dose and shock dose depends not only upon the actual quantities but upon the nature of the antigen involved. Doerr and Russ (26) have shown that with highly active euglobulins shock may be induced by relatively lower multiples of the sensitizing dose than in the case of less antigenically active albumins. In all cases the dose of antigen necessary to elicit shock is considerably larger than the smallest sensitizing dose of the same antigen.

Duration of Hypersensitive State. Mild but definite anaphylactic reactions may be elicited in guinea pigs sensitized with horse serum as long as 245 days after the injection (28), there being no particular difference in this respect between animals sensitized with moderate doses or those sensitized with large doses. A few guinea pigs sensitized with toxin-antitoxin mixtures have shown positive reactions after as long as 732 days, and in one case, reaction was obtained after 1096 days. It is not at all unlikely that individuals once sensitized remain so for life, the sensitiveness gradually fading unless new contact with the antigen occurs. Desensitization, as we shall see, merely interrupts this condition for a limited time, the individual returning to eventual hypersusceptibility.

Passive sensitization consists in transferring the hypersusceptible condition to a normal animal by injecting into it serum containing antibodies.

M. Nicolle (29), in 1907, published a study on the phenomenon of Arthus in which he showed that if the serum of a hypersusceptible rabbit (sensitized with horse serum) was injected into a normal rabbit, the recipient was rendered sensitive, so that the subcutaneous injection of horse serum, made 24 hours later, produced typical infiltrations. Richet (30) soon after this succeeded in transferring hypersusceptibility toward mytilocongestin (a mussel poison) from a sensitized to a normal dog by injecting considerable amounts of the blood from the former into the latter. In this case, too, the hypersusceptibility of the second dog did not appear until one or two days after the injection of the blood. At almost the same time Otto (31) and Friedemann (32) independently succeeded in transferring serum anaphylaxis from hypersusceptible to normal guinea pigs. In the following year (1908) the facts of passive sensitization were confirmed and extended by Besredka (33), Lewis (34), and others (35).

Otto showed that passive sensitization could be carried out with the serum of an actively sensitized animal 8 days after antigen injection, at a period when the animal itself had not yet become hypersusceptible. He also showed that the passive transfer of anaphylaxis need not be confined to animals of the same species, but that guinea pigs could be rendered passively anaphylactic with the serum of sensitized rabbits. Hyper-

susceptibility can be conveyed by the precipitating sera of animals immunized with a foreign protein. This method was later employed by Doerr and Russ (36) in quantitative studies on the relations between sensitizing potency and antibody. Not all immune sera can be used indiscriminately to sensitize other species. Transference is unsuccessful in attempts to use the serum of birds on mammalia, and *vice versa.* White mice cannot be sensitized with the serum of rabbits and guinea pigs. The constitution of the proteins in species far removed from each other seems to render difficult, or perhaps entirely to preclude, combinations of the tissue cells with the foreign antibody globulins.

The principle here involved has extended recently to the explanation of failure of passive sensitization of guinea pigs with anti-bacterial horse serum, and the demonstration that such sensitization can be carried out by similarly immune rabbit sera. Recognition of these facts has cleared up a number of difficulties in the understanding of the mechanism of certain forms of human hypersensitiveness.

Passive sensitization, once established, may persist for as long as 3 or 4 weeks, though Rosenau and Anderson found that animals tested 26 days after treatment reacted but weakly. In the young of anaphylactic mothers Otto has observed positive reactions as long as 44 days after birth, though fatal results were obtained only in pigs a few days old. In general, passive sensitization lasts longer if conferred with homologous rather than heterologous antiserum.

In passive sensitization a definite period must elapse between the injection of the sensitive blood and that of the antigen.

Both Friedemann and Otto found that when the serum of a sensitized animal was injected subcutaneously the best results were obtained by administration of the antigen 24 to 48 hours after this. On intraperitoneal injection of the sensitizing serum Doerr and Russ (36) obtained the best results by permitting an interval of 24 hours to elapse. This period may be shortened to 4 hours by injecting the sensitive serum intravenously.

From these observations the natural deduction was made that the anaphylactic symptoms were the result of cellular occurrences, and that the antigen could act only after the sensitizing substance (however conceived) had become attached to cells. It was thought that a meeting of antigen and antibody in the circulation would result in no reaction. It will be necessary to recur to this problem when we discuss the theories of anaphylaxis.

Inheritance of Anaphylactic Hypersensitiveness. The inheritance of the anaphylactic state may depend upon active sensitization by intrauterine passage of antigen from mother to offspring or may be a passive sensitization by a similar transfer of antibodies. The early observations on such inherited hypersensitiveness were made by Rosenau and Ander-

son and by Lewis (34). Hereditary differences in capacity for sensitization will be discussed in a later section.

The Antibody Involved. Doerr and Russ were the first to develop quantitative methods of studying the so-called anaphylactic antibody. They found that there was an apparently complete parallelism between the ability of an immune serum to sensitize passively, and its content in precipitins. They produced precipitating sera in rabbits in the usual way. With these they passively sensitized guinea pigs, testing them with antigen 24 hours later. To arrive at quantitative results they developed two methods. These consisted in: (1) Intraperitoneal sensitization of guinea pigs with constant quantities of titrated precipitating serum. Twenty-four hours later intravenous test with diminishing amounts of specific antigen. (2) Intraperitoneal sensitization with diminishing quantities of the titrated precipitating serum, and 24 hours later intravenous tests with constant amounts of antigen.

In this way they showed that there was a direct relationship between the power of a serum to convey anaphylaxis passively and its contents of precipitins.

In this, then, we have a quantitative analysis which shows a direct relationship between the concentration of antibodies in a serum and its ability to sensitize passively. Doerr and Russ were inclined, therefore, to identify precipitin and anaphylactic antibody (36, 37). Richard Weil (38) found that a specific precipitate obtained by the precipitation of serum with an antiserum, washed free from serum, produced passive sensitization when injected into a guinea pig. Since such precipitates conveyed active as well as passive anaphylaxis, it seems reasonable to assume that the antigen and antibody, united in the precipitate, have dissociated and in this way produced both effects. The further experiments of Weil in which he removed the precipitating function of a serum by heating at 70° (or by extraction of the antibody from the precipitate with sodium carbonate), without removing its sensitizing functions, are not contradictory to the general identification of these antibodies, since the actual flocculating properties of an antiserum may be physically interfered with by heating.

One of the most discussed objections to the identification of precipitin and anaphylactic antibody has been based on the work of Longcope (39), Spain and Grove (40), and others, who were not able to sensitize guinea pigs with precipitating sera obtained from rats. In view of what we have said above concerning the failure of passive sensitization with certain heterologous antisera, this objection has lost weight.

That there are occasional sensitizing properties of sera which do not precipitate their antigens *in vitro* likewise is a fact which has little significance, since it is apparent from our previous discussions that in order

to precipitate *in vitro* a serum must contain a sufficient concentration of antibodies to coat the surfaces of the finely divided antigenic colloidal particles with antibody containing globulin and thus to render them amenable to the flocculating power of the electrolytes present.

In the discussion on local anaphylaxis the relationship of precipitating antibodies to the Arthus phenomenon has made it clear that there is in such easily measurable reactions a definite relationship between the two serum functions. Observations by Ward and Enders have suggested that complement fixation is a more accurate measure of the sensitizing capacity of a serum than actual precipitation. This we believe to be quite likely, since complement fixation in general is a more delicate measure of antibody contents than visible precipitation.

As a matter of fact, the identification of the anaphylactic antibody with precipitation or any of the other antigen-antibody reactions is of no great importance. The unitarian conception of antibodies makes it necessary merely to demonstrate the parallelism between sensitizing power and antibody contents. Precipitation and complement fixation happen to be the most delicate available methods of titrating antibodies. The basic fact remains that sensitizing power is a function of the presence of specific antibodies.

From what has been said it is clear that there is nothing astonishing in the fact that the serum of a sensitive animal may sensitize when ordinary precipitin or other antibody reactions cannot be easily demonstrated and that an animal may be highly sensitive without such serum constituents. For, in the first place, an antibody content too meager to be determinable by *in vitro* tests may suffice to sensitize and, in the second place, since the anaphylactic reaction is a cellular one there may be sensitizing antibodies on the cells with little or no antibody in the serum.

Symptoms of Anaphylactic Shock. *Guinea Pigs.* If a sensitized guinea pig receives a second injection of an antigen after a suitable incubation time, a characteristic train of symptoms ensues. There is usually a short preliminary period, lasting either a fraction of a minute or several minutes according to the violence of the reaction and the mode of administration. At the end of this time the animal grows restless and uneasy, and often rubs its nose with its forepaws. It may sneeze and cough. At the same time increased respiration is noticeable and the fur appears ruffled. In light cases the animals may remain in this condition, with further difficulty of respiration, discharges of urine and feces; then gradual recovery may set in, with complete return to normal in several hours. In more severe cases these preliminary stages are rapidly followed by weakness. The animals fall to the side, the legs and trunk muscles twitch, and the respiration becomes slow and shallow. The dyspnoea is inspiratory. The excursions of the lung grow shallower in spite of strong inspiratory efforts, the volume of the thorax and lung remaining in the expanded condition. At this stage the

animal may arise and attempt to run. More often, however, general convulsions set in, and in these the animals usually die.

The heart continues to beat for a considerable time after breathing has stopped.

The lungs of such guinea pigs after death are found distended and completely filling the thorax (41, 42, 43). They are pale and bloodless and do not collapse as the pleurae are opened. The alveoli are distended and small hemorrhages may appear upon the serous surfaces. Histological study of the other organs shows hemorrhages in the brain, stomach, heart, cecum, and spleen — more rarely in other organs — and there are local fatty changes in the capillary endothelium. A detailed physiological study of the mechanism of the respiratory death in guinea pigs was first made by Auer and Lewis (44, 45) in 1909. During the later respiratory symptoms, little or no air enters the lungs, although the animal makes violent respiratory efforts. This is due to contraction of the small bronchioles, which occludes the air passages. The origin of this contraction is peripheral. The same phenomena occur in the guinea pigs even after the cord and medulla have been destroyed and the vagi divided. In such cases, of course, with the cord and medulla destroyed, artificial respiration has to be done, and when the symptoms set in it is found that the lungs can no longer be expanded by the same force of artificial respiration which before this had been sufficient.

They showed also that the non-collapsible expansion of the lungs after death was due to imprisonment of the air in the alveoli by the contracted musculature of the small bronchioles, and further confirmed the peripheral origin of this contraction by the discovery that atropin will protect, often preventing death.

Anderson and Schultz (46) found that atropin, methane, chloral hydrate, adrenalin, and pure oxygen will prevent asphyxiation in all but the very acute reactions. The animals may be saved from suffocation in this way, but may nevertheless die, probably as the result of lowered blood pressure.

Biedl and Kraus (47, 48) have confirmed these observations. Schultz (49, 50) studied the intestinal muscle of sensitized guinea pigs excised and suspended in Howell's solution. In this way he showed that during the period of hypersusceptibility the smooth muscle is abnormally sensitive to treatment with the antigen.

In addition to these predominant features in guinea pigs, there are a number of secondary reactions. The fall of blood pressure secondary to preliminary vasoconstriction is followed promptly by vasodilatation of the peripheral vessels. The fall of temperature first described by H. Pfeiffer (51, 51 a) seems to be so constant that it has been taken by a number of workers as one of the necessary criteria of the anaphylactic condition.

Another characteristic of anaphylaxis in guinea pigs is the reduction in the amount of alexin, first noticed by Sleeswijk (52), which occurs during the development of shock. There is a lowered coagulability of the blood, a temporary diminution of the polynuclear leucocytes (leucopenia) and eosinophilia.

The description of anaphylactic shock in the guinea pig which we have given refers to the acute manifestations which occur when highly sensitive animals are injected with fatal amounts of antigen. It must be remembered, however, that all degrees of severity of anaphylactic shock are possible and

that protracted shock, coming on slowly and lasting for a considerable time, may present a clinical picture quite different from the acute variety. In such protracted conditions there may be mild dyspnoea and irregularity of breathing, with partial pulmonary emphysema, fall of blood pressure, and a drop in temperature followed by moderate degrees of fever.

Anaphylactic Shock in Animals Other than the Guinea Pig — General. We mentioned at the beginning of our chapter that one of the characteristics of anaphylaxis is the fact that different animal species react with entirely different trains of symptoms, but that for each particular species the symptoms of anaphylaxis are the same, whatever the antigen.

In all animals, however, there are certain basic physiological changes underlying the anaphylactic mechanism which produce modified symptoms in the individual species, largely because of anatomical and physiological variations and differences in the capacities of these animals to produce or to anchor antibodies.

While we have no positive knowledge of the site of the anaphylactic reaction within the body, it is likely that the primary point of attack is in the reticulo-endothelial system. All varieties of anaphylactic reaction are accompanied by vasomotor changes with peripheral contraction of the arterioles which is probably the cause of the preliminary increase of blood pressure, followed by vasodilatation, with which there is the drop of blood pressure which is a constant feature of severe anaphylactic shock in all animals. Dale and Richards (53) believe that the capillary dilatation is due to direct action upon the capillary endothelium with consequent increased capillary permeability. Whether or not a toxic irritation of the endothelium is involved in this (histamin) is uncertain. Edema occurs in almost all local manifestations of hypersensitiveness, skin reactions, the Arthus phenomenon, etc., and there is at the same time increased concentration in the lymph of blood proteins and of foreign colloids injected during shock. This has been investigated by Petersen and Hughes.

Either as a consequence of the primary capillary endothelial changes or independently there are other physiological phenomena which are fairly constant in anaphylaxis. There is a general irritability of smooth muscles other than the capillaries, *i.e.*, the uterine muscles and intestinal musculature of guinea pigs and dogs. The urinary bladder of most animals contracts. There is marked reduction of coagulability of the blood due to a diminution of fibrinogen. The leucocytes are diminished and there is, in all severe forms, a temporary increase of metabolism and nitrogen output.

Rabbits are not as easily sensitized as guinea pigs. Several preliminary injections are required before fatal shock or the Arthus phenomenon can be elicited. Rabbits are not highly sensitive until a considerable amount of antibody has appeared in the blood. This does not mean that in rabbits the reaction is an intravascular rather than a cellular one. It is probable that in different animals there are differences in the equilibrium between sessile and circulating antibodies and that in guinea pigs there may be a considerable concentration of antibodies in the cells before the serum shows any appreciable concentration, while the condition seems reversed in rabbits.

When a sensitized rabbit is reinjected with antigen a short incubation time is followed by weakness, discharge of urine and feces, and irregular and difficult respiration. This may be alternated with motor irritability,

running spells, and convulsive movements. Death may occur within a few moments or within several hours.

Unlike guinea pigs, rabbits do not show distention of the lungs. There is, however, a characteristic distention of the right side of the heart due to spasm of the arterial side of the pulmonary circulation. Coca found that while a pressure of 10 cm. would easily perfuse a normal pulmonary vascular system, a pressure as high as 90 cm. will fail to perfuse that of rabbits that had just died of anaphylactic shock.

Dogs are not easily sensitized to fatal shock, but after several preliminary injections intravenous inoculation may kill. Anaphylactic dogs after injection grow restless, vomit, and pass urine and feces. This is followed by rapid weakness and labored respiration. Constant characteristics are marked fall in blood pressure and lessened coagulability of the blood which, according to Biedl and Kraus (47, 48, 54), may amount almost to prevention of coagulation. There is, according to Calvary (55), a marked increase of lymph flow which is not dependent upon the low blood pressure, as was shown by controlling the blood pressure with barium chloride. This is of interest in connection with the similarities between anaphylaxis and peptone poisoning, in which Heidenheim noticed a similarly increased flow of lymph. In dogs the anaphylactic reaction seems to concentrate chiefly in the liver. Manwaring showed that when the abdominal vessels were ligated just above the diaphragm, shock did not occur. Exclusion of the spleen, stomach, kidneys, suprarenals, and ovaries from the circulation had no effect, but when the liver was thrown out of circulation, none of his seven dogs reacted with anaphylactic shock. Richard Weil also demonstrated that most of the circulatory disturbances in anaphylactic dogs could be attributed to obstruction of the portal circulation.

Pearce and Eisenbrey (56, 57) attributed the fall of blood pressure to action upon the peripheral vasomotor system. Biedl and Kraus concluded that this action is upon the muscle cells themselves rather than on the nerve endings — because of the fact that adrenalin, which acts by stimulation of the vasomotor nerve endings, does not raise the low pressure in dogs during anaphylaxis, while barium chloride, which acts upon the smooth muscle fibers themselves, strongly raises the blood pressure in such animals. Pearce and Eisenbrey favored the belief that the action is chiefly upon the nerve endings, though both factors, nerve and muscle, may be involved. They worked with apocodein, a substance which, in large doses, paralyzes the vasomotor nerve terminals (58). When a sensitized dog was treated with apocodein and the antigen then injected, no further drop of pressure was obtained. Apparently a paralysis of the vasomotor nerve endings had removed the point of attack upon which the anaphylactic poison could act.

In addition to the symptoms already enumerated, Weichhardt and Schittenhelm (59) claim that anaphylaxis in dogs is invariably accompanied by a severe local reaction in the gut. The intestinal mucosa is swollen and contains miliary hemorrhages, and the lumen is often filled with a mucus mixed with blood.

Manwaring (60, 61, 62, 63) found that if specific foreign protein is injected in the mesenteric vein of an anaphylactic dog, shock-blood collected as it escapes from the liver, and this blood transfused into a normal dog, it reproduces all the characteristic features of canine anaphylaxis. From this he deduced the formation of an anaphylactic poison in the liver. But

since blood drawn from the carotid artery from 1 to 5 minutes after throwing the dog into anaphylactic shock shows little or no toxic action, he believes that the hepatic anaphylotoxin is not the only factor involved.

Manwaring has further investigated perfusions in the canine anaphylactic lung sensitized to horse serum and perfused with Locke's solution followed by Locke's solution containing 0.25 per cent of horse serum. He found in such organs a 75 per cent reduction in the rate of perfusion flow, a non-collapse of the lung when the tracheal clamp was released and the escape of large amounts of fluid from the trachea. He uses this as a demonstration of the marked increase of capillary permeability which he thinks is the fundamental physiologic change in protein sensitization to which all other anaphylactic reactions are secondary.

A similar stasis and marked edema of the intestinal mucosa followed by hemorrhage and necrosis has been observed by Manwaring and his collaborators (64) in the intestine during canine anaphylaxis.

Cats are extremely sensitive to the first injection of foreign proteins, as Drinker and Bronfenbrenner (65) demonstrated. This can only be explained by normal toxicity of these proteins for cats. Cats, however, can be rendered anaphylactic in the same way as other animals. Seastone and Rosenblueth (66) sensitized cats by intravenous injections of dog serum and 21 days later reinjected 0.5 cc. of the same foreign serum. They determined anaphylactic reactions by contractions of the nictitating membrane which they had attached by a small hook directly to a kymograph lever. They obtained at the same time characteristic drops of blood pressure from 160 mm. of mercury to 70 mm. within six minutes. They carried out these experiments on animals in which the superior cervical sympathetic ganglia had been removed, thereby showing that the smooth muscle contraction during shock may occur without the mediation of autonomic nervous impulses.

Rats. Arthus (5) in 1903 claimed that he had produced anaphylactic shock in white rats by repeated injections of horse serum. A number of subsequent investigators (67) failed in sensitizing rats. In 1924, J. T. and Frederick Parker (68) succeeded in obtaining anaphylactic reactions in these animals, but found that the picture of anaphylactic shock in the rat resembles more that of the dog than that of the guinea pig. There was concentration of some of the symptoms in the intestinal canal. They sensitized rats actively, and passively demonstrated typical Dale reactions with the isolated uterus. Longcope (69) was unable to sensitize rats with horse serum, either actively or passively, but was able to demonstrate specific precipitins in the serum. From these findings he concluded that anaphylaxis and precipitin reaction are independent processes. Spain and Grove (40) were likewise unable to sensitize guinea pigs passively with precipitating rat serum. Possibly the failure of passive sensitization of guinea pigs with precipitating rat serum might have been due to the relatively low precipitating value of such serum, but it is also possible that the heterogeneity of the rat serum for guinea pigs is responsible.

Mice. White mice have been actively sensitized to horse serum by Braun (70). Ritz (71) has succeeded with a single injection of a small amount of antigen, and obtained severe anaphylactic shock on intravenous reinjection. Doerr and Russ (36), however, had great difficulty in demonstrating anaphylaxis either actively or passively in mice, a result which may be

referable to the use of the intraperitoneal instead of the intravenous route for injection.

Monkeys are refractory to anaphylactic experimentation. Auer (72) failed to obtain any anaphylactic reaction in monkeys, a negative result which confirmed previous experiments by Rosenau and Anderson (25). We ourselves have failed to elicit uterine strip reaction in macacus monkeys on which a cesarean section was performed two and three weeks after sensitization, but we obtained in one monkey, after prolonged sensitization, symptoms of facial edema and localized reactions which were definitely similar to serum sickness in man.

Kopeloff (73) has published fatal reaction in macacus monkeys by the injection of egg white intravenously and intracerebrally and subsequent intravenous administration of the same antigen in very large doses (10 cc.). He suggests that previous failures were due to insufficient amounts of antigen. He reports, also, the production of local Arthus phenomenon in sensitized monkeys by intracutaneous injections.

Grove (74) succeeded in obtaining feeble but distinct passive local sensitization of ringtail monkeys with human serum from ragweed-sensitive individuals. Straus (75, 76), using rhesus monkeys, obtained more convincing results by the same technique with sera of human subjects sensitive to horse serum, horse dander, cotton seed, fish, etc. Straus was able to sensitize rhesus monkeys to poison ivy by repeated applications of solutions of the specific principle to the skin.

Recognizing as fairly well established that in the three animals most thoroughly studied in anaphylaxis, namely, the guinea pig, rabbit, and dog, physiological reactions and localization in organs vary distinctly, many attempts have been made to explain such differences. In guinea pigs the cause of death resides very largely in the musculature of the bronchioles, in rabbits a similar cause is found in the circulation of the lung, and in dogs the hepatic and splenic circulations seem to be particularly involved. Wells has suggested that anatomical differences may explain these variations. Histological study of guinea pigs has shown an astonishing development of the bronchial musculature, the finer bronchioles consisting almost entirely of muscular tubes. In rabbits "the pulmonary arteries present a remarkable degree of muscular development analogous to that of the guinea pig bronchioles." As far as dogs are concerned, Simonds (77) states that the walls of the hepatic veins of the dogs are different from those of any other animal, again, in showing a very high development of musculature. It is at least a reasonable hypothesis that the differences in these species, in reaction to one and the same mechanism, depend to some extent upon fortuitous differences in the distribution of non-striated muscle. Interesting in connection with Wells's suggestion, is the fact that Huber and Koessler (78) have shown that asthmatic individuals show a hypertrophy of the musculature of the bronchioles which transforms them histologically into a condition not unlike that normally prevailing in the guinea pig, and it is well known that in such individuals severe attacks of an asthmatic nature can be elicited on serum injection.

Protracted Anaphylaxis. In the discussion of the anaphylactic reaction given above, we have dealt chiefly with the severe symptoms of acute shock. It must not be forgotten, however, that these conditions

are the manifestations of profound injury, and the localizations in various organs with which we have dealt are chiefly those which are responsible for death, because these particular sites of injury affect the tissues upon which the immediate continuation of life depends. It is not at all out of the question, however, that other parts of the body may suffer, although their injury expresses itself, especially if the insult is repeated, in degenerations and chronic disease. Chiray (79) and Longcope (80) attempted to approach this problem by repeated administrations of protein in moderate doses to rabbits and investigations of subsequent changes in the kidney. The lesions found were interpreted as signifying subacute anaphylactic injury. Boughton (81) has made observations similar to those of Longcope. It is reasonable to suppose that repeated anaphylactic reactions may cause injury to many organs in which they cannot be observed, by acute reaction.

Desensitization or Antianaphylaxis. In sensitized animals the result of a sufficient dose of antigen, given at the proper time, is death. When the time and quantity are so chosen that instead of death there is merely severe anaphylactic shock, the animals are immediately thereafter in a *refractory condition.* That is, they are no longer sensitive to further immediate injections of the antigen. This observation was made by Otto and by Rosenau and Anderson and was thoroughly studied by Besredka and Steinhardt (82), who named the refractory condition "antianaphylaxis." There is practical significance in this fact, since it suggests methods by which such an antianaphylactic state may be induced in sensitized animals without subjecting them to the dangers of shock. This can be accomplished in a number of ways. The injection of moderate quantities of the antigen at a time just preceding the development of hypersusceptibility will render them refractory to later injections. This preventive administration, however, must be given during the later days of the anaphylactic incubation time. If given too soon after the first injection, it may merely delay its development. Thus if antigen is given by a method and in a quantity which would justify us in expecting hypersusceptibility to be developed at the end of 12 days, we can render the animal "antianaphylactic" by a second administration given, say, on the 8th, 9th, or 10th day. If we give it on the 2d, 3d, or 4th day after the first injection, the only result will be prolongation of the incubation time.

Desensitization appears to be due to partial or complete saturation with antigen of the "antibodies" present on the cells of the sensitized animal. Quantitative relations are therefore important. For, if the dose of antigen is insufficient to saturate the antibodies, partial desensitization only can be expected. And, conversely, the most thoroughly desensitized animal is one that has recovered from a dose of antigen,

almost but not quite fatal. Moreover, since the suddenness of union of antigen with antibody seems to determine the severity of the reaction, any method by which a sufficient amount of antigen can be introduced in a *gradual* manner may serve to desensitize without severe symptoms. This, then, should be the principle in all methods of desensitization. Besredka accomplished it by the repetition of minute doses, whereas Friedberger and Mita (83) accomplished the same purpose by administering the antigen very slowly in dilution; we have already mentioned attempts to desensitize through the gut, a method based on the same principle.

There is no fundamental difference in the desensitization of actively or passively sensitized animals.

In summary, then, desensitization is a specific process by which antigen is administered to the sensitized animal either in sublethal, fractional amounts or in a gradual manner in point of time, so that the reaction takes place without sudden, explosive union. The sensitive animal so treated loses its capacity to react with the antigen for a time. Repeated reactions can be elicited if the first administration of antigen is insufficient to saturate the available antibodies. It is for this reason that it is so difficult to desensitize human beings. Methods of desensitization in man are treated under appropriate headings in a later section.

Desensitization is temporary. In guinea pigs the refractory condition may last two weeks or longer and in rabbits, according to Scott (84, 85), the refractory condition lasts only a short time because of the energy with which this animal produces antibodies. It is plain that even desensitization is, in a sense, a renewed sensitization, since the antigen at first saturates the existing antibodies and secondarily stimulates the formation of new antibodies; eventually, after periods of time depending upon animal species, nature of the antigen, and amounts involved, renewed hypersensitiveness occurs.

Non-Specific Desensitization. There are a number of non-specific methods by which the anaphylactic sensitiveness of an animal can be diminished. Dale observed that the uterus of a guinea pig sensitized against two different antigens and then desensitized completely to one of them loses a certain amount of sensitiveness to the other. Biedl and Kraus (86) in studies on peptone shock found that the injection of peptone into a sensitized guinea pig reduces the animal's sensitiveness to the specific antigen. Karsner and Ecker (87) found that a limited degree of desensitization can be induced in animals by injecting heterologous serum shortly before the homologous antigen is reinjected. Similar observations have been made with anaphylatoxin by Friedberger (88) and by Loeffler. Just how these observations are to be interpreted it is difficult to say. The specific mechanism cannot, of course, be operative.

Doerr suggests that such desensitization depends upon fatigue of the smooth muscle.

A curious fact is the extinction of cutaneous hypersusceptibility against tuberculin and foreign proteins at the time of the rash in measles. As the rash disappears, hypersusceptibility of the skin seems to return. An explanation for this is lacking.

The preventive influence of atropin in anaphylaxis has been noted in connection with the work of Auer and Lewis. Besredka claims to have prevented fatal shock by ether anesthesia on the theory that most of the reactions take place in the central nervous system. It is likely that Besredka underdosed his animals, and by the ether anesthesia merely prevented convulsive symptoms. Banzhaf and Famulener (89) prevented fatal shock by large doses of chloral hydrate. Rosenau and Anderson (90), carrying on similar investigations with urethane, paraldehyde, chloral hydrate, and magnesium sulfate, came to the conclusion that none of these drugs had any noticeable effect on anaphylactic guinea pigs. The most important substances antagonistic to anaphylactic shock are atropin, barium chloride, and adrenalin.

The observation of Friedberger (91) that injections of moderate amounts of hypertonic salt solution would diminish the severity of anaphylactic shock was shown by Zinsser, Lieb, and Dwyer (92) to be due to the fact that the hypertonic salt solution acts by diminishing the irritability of smooth muscle.

Local Protein Anaphylaxis. Arthus (5) in 1903 had found that when horse serum was injected under the skin of rabbits that had been highly immunized and contained a considerable concentration of specific antibodies, the injection was followed within a few hours by edematous swelling which increased in the course of the subsequent 12 or 24 hours, eventually resulting in infiltrations, edema, and, in severe cases, in gangrene. The phenomenon of Arthus was recognized as a form of hypersensitiveness although it was not easily produced in animals other than rabbits. In protein-sensitized guinea pigs, as a rule, intracutaneous injection is followed by a local wheal not unlike that observed in similarly sensitive men. In dogs hardly any skin reactions can be elicited, and the same is true of rats.

Opie (93, 94, 95) has studied the mechanism of the Arthus phenomenon in its relationship to precipitin formation. He finds that it occurs in rabbits with a severity proportionate to the precipitin titer of the serum, which explains why, in order to produce a powerful Arthus phenomenon, it is necessary to inject rabbits repeatedly. The most severe reactions occur when the precipitin titer of the blood ranges between 1–10,000 and 1–50,000. He believed that the precipitate formed by the protein antigen and its antibody is an inflammatory irritant which attracts

leucocytes and does most damage when the precipitation takes place in close contact with the tissue cells.

Since it is difficult to produce a precipitin titer in guinea pigs higher than 1–100, and since neither rats nor dogs are good precipitin producers, this explains why active immunization does not lead to a well-developed Arthus phenomenon in these animals. It does not, however, explain why it is difficult to produce such a reaction in guinea pigs and rats by passive sensitization with a powerful rabbit serum. Reactions can be obtained in this manner, but they are in no way comparable to those of the rabbit. We are inclined to believe that the severest forms of this reaction occur when the antibodies are in considerable concentration on the cells of the actively immunized animal, a concentration which probably cannot be produced by the mere injection of a potent anti-serum.

Opie has also, by means of the Arthus phenomenon, produced what may be spoken of as reversed anaphylaxis. When he injected 1 cc. of horse serum into the ear vein of a rabbit and followed this on the next day, in two different places, with injections of anti-horse serum and of normal horse serum, respectively, the anti-horse serum produced an inflammatory edema, while the normal horse serum gave no reaction whatever. Also, when he injected 5 cc. of horse serum intravenously and followed this in 30 seconds by an intradermal injection of 2 cc. of an anti-horse serum with a titer of 1–100,000, he obtained an extensive inflammatory edema.

Massive injections of antigen do not completely desensitize local reactions, an observation which has importance in its bearing on the difficulty of desensitizing human skin hypersensitiveness.

An interesting observation bearing on the Arthus phenomenon has been made by Auer (96). He was carrying out hypersusceptibility experiments in dogs which had been treated with horse serum and employed heavy doses of horse serum for reinjection. At the site of operation in the inguinal region, he observed — two days later — an extensive edema. He assumed that the local reaction was due to the fact that a foreign protein, horse serum, was circulating in the blood and that a certain amount of this protein passed into the tissues along the wound, and an anaphylactic tissue contact was established.

In human hypersensitiveness there is a marked tendency to local manifestations. This is apparent in the varieties of skin reaction, in respiratory and intestinal manifestations. The most striking analogues to the Arthus phenomenon occurring in man are the indurated areas which are apt to occur at the sites of inoculation after the sixth or seventh injection of rabies prophylactic. In serum sickness, moreover, the accelerated reactions which may occur at the site of injection are, in all essentials, similar to the Arthus reaction.

The Site of the Anaphylactic Reaction. Even in the earliest theories, the possibility of a participation of the tissue cells was suggested. Friedberger (97), as early as 1909, advanced the idea that a precipitation reaction upon the surface of tissue cells, loaded with receptors in the sense of Ehrlich, represented the true mechanism of shock.

The conviction that the meeting of antigen and antibody in the blood stream alone could not account for the condition was forced upon investigators by the observation that passive sensitization was not established until a definite length of time had elapsed after the injection of the sensitizing serum. The regularity with which the necessity for this interval was observed demanded an explanation. Both Friedemann and Otto had found that when the serum containing antibodies was injected subcutaneously, the best results were obtained by administration of the antigen 24 to 48 hours later. Doerr and Russ confirmed this for intraperitoneal injection and found that even when a potent antiserum was intravenously injected, the interval could not be shortened to less than 4 hours.

The simplest explanation presenting itself was that this interval was needed for the attachment of the antibodies to the cells and that hypersensitiveness implied the presence of sessile receptors. The idea itself was not new. It will be remembered that Ehrlich conceived the production of antibody to be in the form of production of specific receptors upon the cells, and that Wassermann had suggested this state of cellular crowding with receptors in explanation of the paradox phenomenon of Kretz, that is, the observation that, occasionally, a horse partly immunized succumbed suddenly to a moderate amount of toxin.

Doerr and his collaborators (36) strengthened this view by showing that the sensitive state in passively treated animals developed progressively as the antibody disappeared from the circulation, and that hypersensitiveness was found most highly developed in guinea pigs when the injected antibody had almost entirely disappeared from the blood.

Manwaring (98) supported the cellular conception in the following manner: He bled a sensitized dog and reinfused him with the blood of a normal dog until there had been an almost complete replacement of blood. He found that the sensitized animal, in spite of the replacement of his blood with normal blood, remained sensitive. Pearce and Eisenbrey (57) carried out a similar experiment, but, in addition, transfused the "sensitive" blood into a normal dog. On reinjection they found that the normal animal, containing the sensitized blood, did not react, but that the sensitized dog, having the normal blood, showed the typical physiological signs of anaphylaxis. Coca (99) carried out analogous experiments in guinea pigs.

Weil (100) found that guinea pigs that had been either actively or

passively sensitized could be partially protected against anaphylactic shock by the introduction of large amounts of specific immune body just previous to the shock injection. He showed, in principle, that if the antigen could be diverted from the cells by antibodies, shock was either prevented or diminished. To obtain such results it is necessary to inject very large amounts of antibody — amounts too large to render this method practically serviceable.

Final proof of the cellular site of the major anaphylactic reaction was brought when Schultz (49, 50, 101) in 1910 introduced the study of the isolated smooth muscle for anaphylactic tests. Using intestinal muscle, he showed that when such tissue was taken from anaphylactic animals, and subjected, in Locke solution with proper kymographic attachments, to contact with the specific antigen, it would react, by contraction, to amounts so small that they had practically no effect upon the normal tissue. The method was improved by Dale (102), who substituted the use of strips of uterine muscle from virgin guinea pigs for intestinal muscle preparations. Dale found that the uterus of an animal sensitized to horse serum would react with a sharp contraction in dilutions of 1–2000 to 1–10,000, while normal guinea pig uteri reached the limit of reaction at dilutions below 1–20. It has not yet been possible to sensitize an excised normal uterus by immersion in antibody solutions.

Weil (101, 103, 104) utilized Dale's method in answering a number of objections to the cellular conception. It had been suggested that the reaction of the sensitized muscle might be due to the fact that the blood had not been entirely washed out of the tissue spaces as was later still maintained by Larson and Bell (105). Weil answered this by administering considerable amounts of anti-horse serum to guinea pigs, killing the animals after a few minutes, then suspending the uteri in Ringer's solution in the usual manner, without attempting to wash them free of blood. Such uteri did not react.

The group of studies just outlined has left no possible room for questioning the cellular localization of anaphylaxis in the sense that anaphylactic shock depends chiefly upon a reaction which takes place specifically between antigen and antibody, either upon the surface or within certain tissue cells.

This accepted, it becomes necessary to explain the occasional exceptions to the necessary interval between passive sensitization and the development of shock.

Weill-Halle and LeMaire (106) reported the development of anaphylactic symptoms in guinea pigs simultaneously injected with antiserum from rabbits and the antigen. Friedemann (107) obtained apparently typical anaphylactic symptoms on the simultaneous injection of antigen and

antibody into opposite ear veins in rabbits. Richet obtained a few similar results in guinea pigs. Biedl and Kraus (108) obtained immediate severe symptoms in guinea pigs by intravenous injections of mixtures of horse serum, together with the serum of sensitized guinea pigs. Briot (109) reported similar observation in young rabbits injected with mixtures of horse serum and anti-horse serum. Gurd (110) and Scott (85) described analogous results.

In this connection the experiments of Kellett are of importance. Kellett was attempting to produce what we may call reversed general anaphylaxis in guinea pigs in a manner analogous to that by which Opie had produced a reversed Arthus phenomenon in rabbits. He injected rabbit-vs.-horse antiserum into guinea pigs previously treated with horse serum. Not only did he obtain occasional acute anaphylactic shock in this manner but in many of his animals shock was obtained when the interval between the two injections was as little as 45 minutes. Zinsser and Enders (113), following the clue furnished by Kellett's experiments, obtained generalized anaphylactic reactions by injecting rabbit anti-horse serum and horse serum into guinea pigs almost simultaneously. It was immaterial which of the two reacting substances was injected first. An analysis of these phenomena, so different in principle from those generally accepted, appeared to indicate that this paradox behavior occurred only in guinea pigs of a given breeding strain. Guinea pigs from only one breeding establishment gave reactions of this kind, whereas other guinea pigs of analogous size and weight showed no symptoms whatever under similar conditions. We are inclined to assume that there may be hereditary variations in the delicacy of that particular mechanism which leads to shock when antigen and antibody unite in the body. The exact nature of this mechanism is still to be determined. Fundamental hereditary variations of this kind may be responsible.

It may be that under certain conditions the injury produced without the interval between sensitization and shock dose has some relationship to the conditions prevailing in local anaphylaxis as described, for the Arthus phenomenon, by Opie (93, 94, 95). It is conceivable that, under proper quantitative conditions, precipitation might occur in the blood stream with consequent disturbance of colloidal conditions and the development of an anaphylactoid reaction (*vide infra*).

These exceptions, which would make it appear that in special conditions the meeting of antigen and antibody in the circulation can cause injury, must be borne in mind, but they do not invalidate the fundamental truth that the important reaction which governs the mechanism of anaphylaxis under ordinary conditions is a cellular one.

There has been much speculation as to the exact site of such cellular reactions. The particular importance of the reticulo-endothelial system, especially the endothelium of capillaries, has been repeatedly suggested in this connection, possibly because these tissues have been regarded as sites of antibody formation.

Vanucci (114), Gay and Clark (115), and others have found that antibody formation could be diminished in animals by saturation with trypan blue,

which is selectively stored by the reticulo-endothelial cells. Results, however, have not been uniform. Standenath (116) and others failed to block anaphylactic shock in sensitized animals by preliminary trypan blue inoculations. Klinge (117, 118), claimed that he completely prevented the development of the Arthus phenomenon in sensitized rabbits by introducing the antigen into a tissue area which had been locally blocked by considerable doses of trypan blue. Jungeblut and Newnan (119) blocked guinea pigs by intravenous injection of both India ink and trypan blue and then injected them with a toxic, heterophile immune serum, finding that death was slightly delayed. Trypan blue completely inhibited the skin reactions which in normal animals follow the intracutaneous injection of the Forssmann serum.

Blocking experiments, while inconclusive, suggest that interference with the normal function of the capillary endothelium may inhibit both antibody formation and anaphylactic response.

Petersen and Levinson's (120) studies on the alterations of the lymph in sensitized animals reinjected with proteins also indicate a sudden stimulation and increased permeability of the endothelium.

The other tissue which seems primarily affected in anaphylactic shock is smooth muscle. This is the basis of the Schultz-Dale reaction and is apparent in the vascular changes just discussed. It may, of course, be that the smooth muscle reactions are secondary to preliminary action upon endothelium.

This does not necessarily indicate that the actual meeting of antigen and antibody must take place upon the endothelial cells themselves. It is not inconceivable that the reaction, taking place elsewhere, liberates toxic substances or produces changes which secondarily act upon the capillaries. Manwaring from his studies in dogs suggested that in passing through the liver the antigen causes the liberation of an anaphylatoxin-like substance to which the other manifestations are secondary.

As to the actual factors of injury to which anaphylactic shock is due, there are, in the main, two lines of reasoning which may be classified into the *physical theories* and the *chemical theories.*

The *physical theories* of anaphylaxis assume (121) the occurrence of colloidal changes in which fibrin is formed upon the cells, interfering with metabolism. The views of Jobling and Petersen (122) involve physical changes in the blood stream secondary to which there is a formation of a toxic substance. Colloidal changes in the plasma bring about a rapid metabolism by non-specific protease, a decrease of anti-ferment, and an intoxication due to the cleavage products. Jobling and Petersen's theory does not give due consideration to the cellular site of the reaction. Doerr (123) suggests that the reaction between antigen and antibody involves the cell membrane, as a consequence of which there is cell irritation. Wells (124) expressed a similar view when he stated that "slight disturbances in the equilibrium of plasma colloids render them highly toxic and that similar alteration in colloidal equilibrium within the cell protoplasm may produce equally profound intoxication of the cell."

Other theories have largely considered the possibility of the formation of a toxic substance as a consequence of the meeting of antigen and antibody.

The most important toxic substance discussed in connection with anaphylaxis is *histamin*. Dale and Laidlaw (125) found that histamin, which is a histidin derivative, when injected into animals, causes symptoms similar to anaphylactic shock. It produces fall of blood pressure, bronchial spasm in guinea pigs, the pulmonary-circulatory obstructions in rabbits, and local skin changes in human beings. Moreover, as Wells states, histidin, from which it is derived, is present in every known complete protein. Wells points out that some other protein cleavage products, such as methyl guanidin, have physiological actions similar to those of histamin. Histamin also stimulates the sudden contractions of the isolated guinea pig uterus in a manner analogous to the contraction of the sensitized uterus upon contact with the antigen.

The conception based on the formation of histamin, then, would be as follows: As a result of the union of antigen with cellular antibody, a chemical change is initiated as a result of which a histamin-like substance is liberated which has powerful irritating effects and gives rise to the vascular and smooth muscle changes underlying the physiological mechanism of anaphylactic symptoms.

Wells (126) lists as the chief objections to histamin its failure to desensitize animals, its production of strong reactions in a specifically desensitized uterine strip, and its failure to produce the coagulation changes usually present in anaphylactic blood.

Yet, though the histamin hypothesis does not fit all observed conditions, there is evidence to support the operation of some similar toxic material in a variety of anaphylactic manifestations. Lewis and Grant (127), studying the urticarial wheals produced in the skin by stroking, found that the fluids obtained from such wheals stimulated uterine contractions in the same manner in which this is done by histamin in high dilution. They studied a patient hypersensitive to fish. Skin tests carried out on this patient with dilutions of histamin and with extracts of fish produced exactly similar reaction both in points of time and course. Hare (128) confirmed these observations and claimed that histamin desensitized the skin locally to the specific reaction and *vice versa*, a fact in contradiction to most other observations on histamin desensitization. Lewis (129) basing his views largely on studies of reactions of the skin, conceives the conditions as follows: The injected antigen reacts with the cells, as a result of which an "H-substance" analogous to, if not identical with, histamine is liberated and this exerts an irritating action on the capillary vessels, dilating them and increasing their permeability. Lewis regards the contraction of the unstriped

muscles and the dilatation and increased permeability of small vessels as the basic phenomena of anaphylaxis.

Best, Dale, Dudley, and Thorpe * have shown that histamine can be isolated from tissues, and their methods suggest that histamine exists preformed in the organs. Watanabe † has shown that there is a marked reduction of tissue histamine in the lungs of guinea pigs and the livers of dogs immediately after anaphylactic shock. The experiments of Dragstedt and Meade ‡ suggest that a substance with toxic action like histamine appears in the blood of dogs after anaphylactic shock. They sensitized dogs with horse serum and after incubation periods of 14 days or longer they anesthetized the animals and inserted cannulae for the collection of thoracic duct lymph for recording of carotid blood pressure and femoral vein injection. The animals were then shocked and samples of blood and lymph collected. These samples were then tested for histamine activity either by the method of the isolated intestinal strip of guinea pigs or by flow of blood pressure by intravenous injection into cats. They compared the effects with those of various solutions of histamine and found that blood samples were obtained which were equivalent to 1–100,000 histamine solution and lymph samples up to 1–150,000. These amounts, distributed over the whole body, would not account for the total effects of shock, but the same writers believe that they have evidence that some of the liberated histamine has been bound.

Anaphylactoid Phenomena. By anaphylactoid phenomena we characterize a group of reactions which in symptomology resemble those of true anaphylaxis but which cannot be referred to a specific antigen-antibody mechanism. The term has been loosely used to include a great many different phenomena.

Toxic Action of Normal Serum. It is well known that the blood serum of some species of animals is highly poisonous for other species. Thus goat serum injected intravenously into guinea pigs and rabbits gives rise to symptoms that simulate anaphylaxis and often lead to acute death. The most toxic of such foreign serum is eel serum, which kills guinea pigs within a few minutes.

The older observations of this phenomenon were made as early as 1666 in a report of the Commission of the London Royal Philosophical Society.

The cause of death following injections of foreign whole blood, blood cells, and serum has, since that time, occupied the attention of many workers Morgagni, Brown-Séquard, Magendie, Naunyn, Landois, and Ponfick.§

Landois attempted to correlate the occurrences *in vivo* with the action of the serum upon the cellular blood elements *in vitro*. He was led to regard the action of serum upon erythrocytes as the primary cause of death after transfusion. His conception is on the one hand a formation of fibrin initiated in the stroma of the injured erythrocytes which leads to coagulation, and on the other hand embolism in the pulmonary vessels due to "Massenhafte Verklebung der Kaninchenzellen im Hundeblut" — or hemagglutination.

* Best, Dale, Dudley, and Thorpe, Jour. Phys., 62 : 397, 1927.

† Watanabe, Zeitschr. f. Immunitätsf., 72 : 50, 1931.

‡ Dragstedt, C. A., and Meade, F. B., Jour. Pharm. and Exp. Ther., 57 : 419, 1936.

§ A brief historical review of this work can be found in the paper of Coca (130).

Ponfick and others disputed the validity of Landois' conclusions, but the basic principles of his explanations have been upheld by others. Coca (130) concludes that death is due to mechanical obstruction of the pulmonary circulation by agglutination of the cells.

Loeb, Strickler, and Tuttle (131) have come to similar conclusions by the use of hirudinized foreign blood.

Uhlenhuth (132), on the other hand, attributes this action to a "complex process depending upon the cooperation of complement," but not identical with the hemolytic mechanism.

Studies of one of the writers (133) on the toxic action of goat serum for rabbits have shown that, even when hemolytic effects are excluded, the foreign serum may be toxic for the tissues of the injected animal.

Thus, the toxic action of normal foreign serum may be due to a number of causes. In some cases death may be due to thrombosis and embolism due to hemagglutination. In others, however, or together with the agglutination phenomenon, there may be a process analogous to anaphylaxis.

Heterophile Antisera as Toxic Agents. In the chapter on Antigens we have discussed the Forssmann Heterophile antigens, and it will be remembered that these antigens are absent from the bodies of certain animal species — rabbits, cattle, etc. — but are present in guinea pigs, horses, sheep, chickens, dogs, etc. Doerr and Pick (134, 135, 136) have shown that if rabbits are injected with blood or tissue extracts containing the Forssmann antigens, sera are obtained which possess primary toxicity for guinea pigs and other animals whose tissues contain the antigen. The clinical and pathological picture in a guinea pig killed by such heterophile antiserum is not readily distinguishable from true anaphylaxis, though the injury is more widely distributed in the organs. Again, we are dealing with a capacity of union of an antigen, a normal part of the cellular structure, with an antibody. In fundamental respects, therefore, the phenomena are comparable to anaphylaxis.

The Toxic Action of Hemolytic Sera. Sera which contain hemagglutinin or hemolytic properties for the blood cells of another species, whether normally present or acquired by immunization, are toxic when injected into an individual of a species whose blood cells they act upon. The problem here is similar to that concerned with the toxicity of normal serum. It may be that besides containing hemolytic and hemagglutinating properties, the sera have in addition the power to react with fixed tissue cells of various types. Symptoms and death can often be explained by hemagglutination followed by embolism.

Peptone Shock. When peptone is intravenously injected into animals, it gives rise to symptoms difficult to distinguish from true anaphylaxis. This was studied in dogs by Biedl and Kraus (57), who showed marked similarities between the two conditions. Recovery from either one of them conveyed a certain desensitization against the other. Manwaring (137, 138), states that the two conditions, though similar, are distinct, because a fall in arterial blood pressure is produced by the intravenous injection of peptone in dehepatized dogs — a condition which is not true of anaphylactic shock.

Miscellaneous Anaphylactoid Phenomena. Anaphylaxis-like symptoms can also be observed when a number of inert substances are injected intra-

venously into animals. Among them are weak suspensions of agar, gum acacia, kaolin, starch, certain organic dyes, collargol, etc. Substances of this kind, covering the widest range of chemical constitution, have in common chiefly their colloidal condition. It seems that the toxicity of such agents must be related in some way to the physical state, depending upon the injection of masses of very finely divided particles which may affect coagulation phenomena perhaps by surface tension conditions. Hanzlik and Karsner (139, 140, 140 a, 140 b, 140 c, 141), conclude that most of the agents mentioned injure by reason of their effect upon the circulation and have no mechanistic relationship to true anaphylaxis.

Anaphylatoxins or Serotoxins. The underlying idea which led to the study of the so-called anaphylatoxins is found in the original endotoxin theory of Pfeiffer. According to this, the action of specific lysin liberated from bacteria a preformed poison, the endotoxin. In 1902 Weichhardt (142), bearing this conception in mind, subjected syncytial protein of rabbit placenta to the action of specific antisera and obtained substances toxic for normal rabbits. The production of toxic substances by specific cytolysis was further investigated by Wolff-Eisner (143) in 1904.

The most important of the earlier investigations along these lines was the work of Vaughan and Wheeler (144). Their conception takes root in earlier investigations of Vaughan (145, 146, 147) upon the extraction of a poisonous group from the protein molecule.

Vaughan and Wheeler (148, 149) were able to split egg albumen and other proteins by treatment with absolute alcohol (containing 2 per cent NaOH) into two fractions -- a toxic alcohol-soluble and a non-toxic alcohol-insoluble one. Injections into guinea pigs of the toxic fraction produced symptoms not unlike anaphylaxis — but did not sensitize against protein. The alcohol-soluble portion was non-toxic and sensitized against protein in doses of 0.001 to 0.005 gm.

Any conception based upon the assumed cleavage of an antigen by antibody action necessarily suggests the possible participation of alexin or complement. Sleeswijk (52) found that there was a diminution of alexin in the blood of an animal immediately after anaphylactic shock. His results were confirmed by Friedberger and Hartoch (91).

This observation raised the question whether the animals die because of a sudden loss of circulating, physiologically necessary alexin, or whether the alexin takes an *active* part in producing the conditions which cause death.

Either of these possibilities might explain the fact of alexin diminution, but the former — the possibility that complement depletion is the cause of death — was ruled out by Friedberger and Hartoch (91). Supplying fresh complement to sensitive animals at the time of reinjection did not prevent shock. Utilizing the fact observed by Nolf (150), Hektoen and Ruediger (151), that hypertonic salt solution (1.5 to 2 per cent) will prevent the combination of complement with its sensitized cells, they slowly injected into sensitized guinea pigs 0.3 cc. of concentrated NaCl solution just before the injection of antigen, and found that these animals survived injections which invariably killed the controls. The value of this experiment for the complement theory has been largely neutralized by work of Zinsser with Dwyer and Lieb (92), in which it was shown that the effect of the salt renders smooth muscle less irritable.

Loeffler (152), using guinea pigs sensitized with horse serum, completely depleted their complement by injecting intraperitoneally considerable quantities of sensitized sheep corpuscles. Tested by injection of horse serum one hour later no anaphylaxis occurred, while controls regularly succumbed. This experiment may now be interpreted as an example of non-specific partial desensitization.

Speculations regarding the participation of complement in the production of an anaphylactic poison then led to attempts to produce the anaphylactic poison *in vitro*. Ulrich Friedemann (107) allowed alexin to act upon sensitized beef blood, interrupted the action by cooling at a time just preceding the occurrence of hemolysis, and injected the supernatant fluid into normal rabbits. The result was illness resembling anaphylaxis. He expressed the opinion that the poisons were formed by the action of "amboceptor" and complement.

Friedberger (153, 154, 155) produced similar poisons by the action of alexin on specific precipitates and concluded that anaphylactic shock is an intoxication due to a poison produced from the products of a precipitin-precipitinogen reaction by the action of complement; he spoke of the poison as *anaphylatoxin*.

The assumption that the poisons were produced by decomposition of the antigen was, however, rendered untenable by the experiments of Keysser and Wassermann (156, 157), who substituted insoluble substances like barium sulfate and kaolin for the antigen; and found that if kaolin were treated with horse serum and then exposed to the action of guinea pig serum or complement, poisons were produced identical with those produced by Friedberger. The conclusions they drew were that the poisons were produced by action of the complement on the horse serum absorbed by the kaolin. Bordet (158) then showed that poisons similar to those of Friedberger could be produced by the action of fresh guinea pig serum on agar even when the agar has been deprived entirely of nitrogenous components, proving that the proteolytic action of the guinea pig serum must have been active against its own proteins.

Jobling and Petersen (159) showed that bacteria mixed with fresh active serum adsorbed the antienzymes normally present in the blood. Similar antienzyme removal can be accomplished by the addition of kaolin or agar, and by treatment with chloroform. Serum so treated becomes toxic, the poisons formed showing great similarity to Friedberger's anaphylatoxins. According to them, the poisons are formed because antienzymes are adsorbed by the antigen, thus setting the normal ferments free to act on their own serum protein.

Novy and DeKruif (160) produced toxic substances by incubating guinea pig serum with trypanosomes and with agar, and found that with this serum, as well as with rabbit and rat serum, poison production took place with great speed. The physical condition of the agar had important influence upon the speed of the reaction. When the agar was finely divided, toxic products were obtained in less than five minutes. 0.0025 mg. of dried agar was enough to toxify a cubic centimeter of serum. This confirmed the conception that substances, such as bacteria, agar, etc., added to sera, acted indirectly in bringing about changes in the serum rather than in furnishing a material upon which the serum enzymes could act. Doerr and Moldovan noticed that in the course of interrupted coagulation, blood may become

toxic. Blood apparently becomes toxic in the stages just preceding coagulation. According to Novy and DeKruif, when the blood is drawn, catalytic substances form which change the fibrinogen into fibrin and produce anaphylatoxin-like substances in the process. These observations are of importance in invalidating such experiments as those of Friedberger, Thiele, and Embleton (161, 162), who tried to prove the existence of toxic substances in the circulation of animals by injecting fresh blood from one animal into another.

As a result of all the foregoing studies on the so-called anaphylatoxic substances we are forced to the conclusion that a great many processes associated with the contact of blood plasma with foreign materials may give rise to toxic products. This occurs easily *in vitro*, and may occur *in vivo* as well, and the poisons thus produced give rise to symptoms not unlike those of anaphylaxis. "Anaphylatoxins" may have importance in general pathological physiology. But it is quite unlikely that these poisons play any role whatever in the phenomena of hypersensitiveness.

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CHAPTER XIII

HYPERSENSITIVENESS, *Continued.* CLASSIFICATION. PRINCIPLES OF HYPERSENSITIVENESS IN MAN

DISCUSSION of phenomena of *Hypersensitiveness* has been rendered more difficult than necessary by a confusion of terms. The original word "anaphylaxis," coined by Richet, was for a time applied only to those forms of protein hypersensitiveness in which circulating antibodies made it possible to fulfill all the criteria of passive sensitization. The word "allergy," introduced by von Pirquet, had at first a general significance for all forms of hypersensitiveness, but in the course of premature classification was loosely applied to those forms of hypersensitiveness which could not, by demonstrable mechanism, be incorporated in true anaphylaxis. The word "idiosyncrasies" has been loosely applied to respiratory, food, and drug hypersensitiveness. There is very little logic in the manner in which the various terms have been used. Since we believe that in all forms of specific *Hypersensitiveness* the same fundamental biological reactions are involved, we will use this general term throughout as signifying the conditions as a whole. We believe it justified by usage to reserve the term "anaphylaxis" for protein reactions in animals in which circulating antibodies play a role. The words "allergy," in regard to phenomena such as the tuberculin reaction, and "idiosyncrasy" as applied to drug hypersensitiveness we retain as having purely the colloquial value of established habit.

Early studies on hypersensitiveness were carried out almost exclusively with reactions in which the incitant substances were native, undenatured proteins such as horse serum, egg white, etc. And with such reactions the basic principles of what is known as "anaphylaxis" were elucidated. It soon became apparent, however, that there were many instances of specific hypersensitiveness, especially those observed in man, in which the responsible incitants were not antigens in themselves and in which a basic mechanism of antigen-antibody reaction could not be determined. In consequence, there was a period of considerable confusion during which the formulation of more or less artificial classifications obscured the fundamental biological principles which govern all these conditions. Much of this temporary confusion was due to inade-

quate information and to the domination of immunological thinking by prematurely crystallized ideas of the nature of antigenic substances. Moreover, the problem was complicated by differences in the manifestations of hypersensitiveness observed in individual animal species. Questions of heredity, heterologous passive transfer, localization of symptoms, sensitization *in utero*, through respiratory and intestinal tracts had been so little investigated that needless differentiations were established on the basis of purely secondary factors.

The most important source of misconception was too narrow a definition of antigens. Until quite recently the capacity to produce detectable antibodies in the circulation of an animal was regarded as the sole criterion on which a substance could be classified as an "antigen." We have learned, however, that a rational comprehension of immunological processes necessitates an inclusion in the biological conception of antigenic substances all materials which, in contact with the animal body, lead to a specifically altered reaction capacity. The studies of compound antigens, and the demonstration that, within the animal body, nonantigenic substances of low molecular weight may combine with proteins to form true antigens, have clarified the mechanisms of forms of hypersensitiveness such as those caused by dyestuffs and drugs. In bacterial allergies like the tuberculin reaction, there are involved nitrogenous, probably protein, materials which induce a high degree of specific hypersensitiveness without ever inducing antibodies in the circulation with which the conditions can be passively transferred.

In order, therefore, to approach problems of hypersensitiveness in a biologically sound manner, it is first necessary to recognize that the presence of circulating antibodies is merely a secondary process characteristic of certain antigenic substances like native proteins and whole bacteria, but that many other substances may produce specific cellular changes of reaction capacity and hypersensitiveness without the appearance of free antibodies. It is more than probable that such differences between the physiological reactions to the inciting substances depend upon chemical and physical differences in the antigens, but that in all cases the fundamental fact of specifically increased cellular reaction-capacity serves to characterize all these phenomena as dependent upon fundamentally similar biological processes. For a more extensive discussion of the description of antigens we refer to the chapter devoted to this subject.

In the chapters which follow we will endeavor to analyze the various manifestations of hypersensitiveness in which these variants play a role.

There are still many uncertainties in our knowledge of hypersensitiveness and for this reason we believe that the rigid classifications which have been suggested are premature. On the basis of the principles which

we have discussed, however, it appears justified to subdivide all forms of hypersensitiveness into two main classes:

I. Hypersensitiveness in which an antibody mechanism is involved.

II. Hypersensitiveness in which so far no evidence of anything analogous to antibodies has been obtained.

I. Hypersensitiveness in which antibodies or functionally antibody-like substances can be shown.

I A. Hypersensitiveness to unaltered proteins against which circulating antibodies are formed. In this class passive sensitization with the sera of actively sensitized animals can be accomplished at any time at which the antibodies appear in the blood. The passive transfer of such specific sensitization tested by the isolated uterus technique of Schultz-Dale is probably the most delicate method available for antibody determination. Passive transfer in such cases is limited to some extent by heterogeneity of species between the donor and the recipient of the sera. In many cases passive transfer from rabbits, man, and other animals to guinea pigs is possible. In other cases — such as, for instance, the transfer of hypersensitiveness to pneumococcus carbohydrate — passive transfer is possible to guinea pigs with antipneumococcus serum from rabbits but not with that of horses.* It may be that such limitations depend upon the chemical differences between the antibody globulins of one species and the cellular proteins of another which prevent the association of the two for the accomplishment of sensitization.

I B. Hypersensitiveness to compound antigens such as the protein-carbohydrate complexes of many bacteria in which active sensitization can be accomplished with the whole antigen but not with the carbohydrate fraction alone and passive transfer is possible with the sera of animals so sensitized. But the sensitized individual, in these cases, will react not only to the whole antigen itself but to the specific haptene factor. These observations have added much to our understanding of bacterial hypersensitiveness and are promising to explain many formerly obscure phenomena concerned with drug idiosyncrasy.

I C. Hypersensitiveness chiefly observed in man, in which antibody-like substances are formed but in which only local homologous transfer is possible — that is, from man to man — by the method of Prausnitz and Küster. In these cases it is still a question whether the antibody-like substances of the sensitizing serum which have been spoken of as "reagins" are fundamentally different from ordinary antibodies, or whether the limitations of transfer are due merely to low concentrations of antibodies and the necessity for strict species identity between donor and recipient (*vide infra*, page 385). At any rate, the Prausnitz-Küster reaction has been obtained with a wide variety of incitant substances such as plant pollen, egg white, various species of meat and fish, horse dander, cow's milk, and many other substances which are either antigens in the old sense or can be made antibody-producing by intensive treatment. While it is convenient,

* Studies by Bailey, Raffel, and Dingle (1) indicate that a quantitative factor may be involved in these relations. They were able to sensitize guinea pigs passively to pneumococcus carbohydrate with anti-pneumococcus horse serum only when very small quantities of the antiserum (0.001 cc.) were used. Larger amounts failed to sensitize.

therefore, to segregate this type of reaction for purposes of discussion, there is no reason at present to regard it as fundamentally different from the preceding.

I D. Hypersensitiveness to substances of low molecular weight (formaldehyde, iodine, coal tar compounds, various organic drugs, and even metallic salts) which are in themselves in no sense antigenic. In many of these cases, such as, for instance, formaldehyde and iodine hypersensitiveness, we have clear evidence that indicates that antigenic compounds specifically oriented by the non-antigenic groups may be formed in the body by combination of the extraneous materials with body proteins. These phenomena may form a basis for a general understanding of drug idiosyncrasy. It must not be forgotten, however, that there are many forms of drug idiosyncrasies in which substances — chemically more complex than, for instance, formaldehyde — are involved in which no direct evidence for the formation of true antibodies has as yet been discovered (see below).

II. Hypersensitiveness in which no antibody mechanism has been so far demonstrable either *in vitro* or by passive transfer.

II A. Hypersensitiveness to certain nitrogenous, probably protein substances (tuberculin, mallein, abortin, etc.), in which active sensitization is in some manner associated with the reactions which take place in the infected animal body between the living or dead organisms and the body of the host. Sensitization in these cases has not been accomplished satisfactorily by the injection of soluble extracts or chemical fractions of the respective bacteria. In these cases, moreover, known as the "bacterial allergies," no passive transfer has so far been clearly demonstrated.

II B. Hypersensitiveness to certain organic compounds and drugs such as dinitrochlorobenzene and a number of other chemically definable substances, quinine, etc., to which human beings become sensitive by prolonged contact and to which guinea pigs can be actively sensitized but in which no evidence of antibody formation has as yet been discovered. This group is segregated in justice to experimental facts although the study of the subject has not been experimentally completed. In most cases this type of drug idiosyncrasy in man occurs in the form of skin manifestations, and animals are most easily sensitized by way of the skin. It is quite obscure at the present time why certain substances like formaldehyde should so readily fall into the class of true antigen-antibody manifestations and in others the antibody mechanism should seem to be lacking.

It will contribute to an understanding of our subsequent discussions if we think of variations in the manifestations of hypersensitiveness as fundamentally dependent upon chemical and physical differences in the antigens. One is tempted to formulate these differences as depending to some extent upon the molecular sizes of the inciting materials, antibody formation resulting only when reactions between the larger complexes and the cells are surface phenomena — that is, extra-cellular — and purely cellular reactions occurring when the association is an intracellular one. This, however, for the present remains pure speculation.

Hypersensitiveness in Man. General Principles. In protein hypersensitiveness of animals it is postulated that the reacting cells are

associated with or contain specific antibodies which render them capable of abnormally rapid and quantitatively excessive reaction with the antigen when this reaches them. Just as the antigen reacts specifically with its antibody *in vitro*, the antigen now reacts with the antibody in or upon the cell surface.

We have seen, however, that even with a similarity of basic mechanism admitted, no two species of animals display exactly the same type of symptoms of hypersensitiveness. This may be due, in part, to anatomical differences such as the varied distribution of smooth muscle, in part to physiological differences which have not been clearly ascertained. That the differences exist, however, is clear from the ease or difficulty with which various animal species can be sensitized and from the differences of manifestation which are species characteristic.

Opinions which favored a fundamental separation of human hypersensitiveness from anaphylaxis in animals were based primarily on the following postulates: Human hypersensitiveness often seems to occur without traceable previous exposure to the incitant substances; the substances which incite human hypersensitiveness are often non-antigenic in the sense that they are incapable of inciting antibody production or of sensitizing animals; in many types of human hypersensitiveness passive transfer is not possible; there is a somatic hereditary factor which determines human hypersensitiveness according to Mendelian formula independent of sensitizing exposures.

There are other secondary factors which have influenced such reasoning, but these are the most important. We will consider them in principle before we proceed to the discussion of individual forms of human hypersensitiveness.

In man there is *little tendency* to acute generalized shock. In this respect the conditions in human beings are similar to those observed in dogs and in monkeys. We have no doubt that the acute shock which can be produced in dogs by suitably repeated injections could probably be produced in man, were we in a position to carry out such drastic experiments; and although most of the acute deaths observed in man after injection of foreign proteins of various kinds are probably more closely related to thymus death and similar phenomena than to anaphylaxis, occasional severe systemic reactions to horse serum in sensitive individuals support the above supposition.

Furthermore, there is in human hypersensitiveness a considerable *tendency to localization* of manifestations. These may be classified largely into the *Respiratory*, the *Intestinal*, and the *Skin groups*. To some extent, this localization may be due to the fact that the natural sensitization of man takes place either by ingestion, by inhalation, or by skin contact. The tissues, therefore, through which the antigen repeatedly

enters may be sensitized more intensely than the rest of the body, or — if the body is sensitized throughout — the tendency which the antigen has repeatedly to enter by a special path will bring the new antigen into most concentrated contact with the tissues in these locations, where — consequently — the most intense reactions are to be expected.

Among the human forms of hypersensitiveness many are incited by true protein antigens, and in these there seems little reason to doubt that the basic mechanism is one entirely analogous to that of protein anaphylaxis. This is true, we believe, of serum sickness and of many food idiosyncrasies. But it is important to note that in symptomatology, localization, skin reactions, and desensitization phenomena these human conditions incited by protein antigens do not essentially differ from those in which the antigenic nature of the inciting substance is not yet established.

When, moreover, *the question of passive transmission* as indicating an antibody mechanism is taken as a criterion of similarity or difference from anaphylaxis, we must remember that the advances of recent years have revealed facts which diminish the negative value of past failures of passive sensitization. The investigations of Tomcsik and Kurotchkin (2), now fully confirmed by Avery and Tillett (3) and by others (4), show that passive transfer depends not only upon the presence of sensitizing antibodies, but also upon the species of animal furnishing the serum. Thus, for instance, guinea pigs cannot be made passively sensitive to certain antigens with immune horse serum, but can be sensitized with immune rabbit serum. Since the antibodies are contained in or closely associated with the serum globulins, and since sensitization implies the establishment of a relationship between these antibodies and the tissue cells, it is quite reasonable to assume that the cells may easily become associated with some animal globulins and not with others. Thus, failure to transfer a human idiosyncrasy to a guinea pig or to fulfill the criterion of the isolated uterus experiment would not necessarily mean that the serum of the individual could not have transferred the idiosyncrasy to a more closely related species. And, indeed, the Prausnitz-Küster reaction has shown that many human idiosyncrasies can be passively transmitted from man to man — homologously, when heterologous transfer succeeds only in exceptional cases and often unconvincingly.

Again, it must be remembered that both anaphylaxis and the human "atopies" or "idiosyncrasies" are cellular reactions, the circulating anti-substances being expressions of the cellular condition and not necessarily present in the serum at all times. Even in true anaphylaxis an animal may be highly sensitive without containing demonstrable antibodies in its blood. And hypersensitiveness may last a lifetime

while antibodies disappear from the circulation in a relatively short period.

Of great importance, furthermore, in appraising evidence in questions of human hypersensitiveness is the recently expanded knowledge of *haptenes or partial antigens.* In the Forssmann antigen, in Landsteiner's compound antigens as in the bacterial antigen, we have examples of substances in which the whole antigen only is capable of inciting antibody production, but in which specific reaction of the immune serum occurs in contact with the haptene or haptophore group, although the latter is quite incapable of inciting antibodies by itself. Thus, for instance, in anaphylactic experiments with the carbohydrate haptenes of bacteria which, though highly specific, are entirely without the ability to incite antibody production, typical systemic response as well as uterus reactions can be obtained in passively sensitized animals.

The problem of hypersensitiveness cannot be intelligently approached without reference to all these complicating factors. Yet enough knowledge has already accumulated to render untenable those views which assert the complete separation of causative and operative mechanisms of anaphylaxis in animals and certain forms of hypersensitiveness in man. We shall hope to make clear that all forms of hypersensitiveness represent essentially one and the same basic phenomenon when analyzed with understanding of superficial variables.

The Spontaneous Sensitization of Man. One of the most important problems is that of the methods by which human beings become spontaneously sensitized. When sensitiveness exists, it is often difficult to determine whether contacts have taken place previous to the first observed reaction, and this supposed sensitiveness upon first contact was the leading motive for the formulation of a theory in which heredity played such an important role. However, in a great many human "idiosyncrasies" relationship between previous exposure and the development of the condition have been shown. Thus in the industries, hypersensitiveness to quinine, to varieties of dandruff, to ipecac powder, etc., have been shown to develop in workers by habitual contact. Rackemann (5) has described azofuchsin hypersensitiveness which developed in a chemist three years after he began working with this material. Sensitiveness to malt, wheat, and hops, asthma caused by leather dust, hypersensitiveness to rabbits and guinea pigs, and to wheat — instances of all of these conditions have been traced to prolonged contact with the responsible substances. Similar contact development of hypersensitiveness to orris powder, and a number of horse asthma cases have been recorded. In drug idiosyncrasies prolonged contact with mercury, arsenic compounds, veronal, and aspirin has preceded hypersensitiveness. Ratner (6) has described the development of rabbit hair asthma in

children who have slept on pillows of rabbit hair, and similar cases are scattered throughout the literature in sufficient number to establish satisfactory evidence in favor of the possibility of actual sensitizing contacts.

In the section of drug hypersensitiveness we shall see that even in the case of simple chemical substances both clinical and experimental evidence unite to uphold the general laws of an immunological mechanism in the development of human sensitization. It is not at all surprising that it is impossible to trace all cases of human hypersensitiveness to the sources of specific exposure if we consider the length of life of human beings and the infinite variety of possible sensitizing substances to which they may have been exposed. Proven instances of actual sensitization for a variety of antigens are therefore of fundamental importance. And the force of the negative argument of failure to trace actual exposure in any given case is much weakened by the fact that the sensitization of man can take place *in utero* through the skin, respiratory passages, and the intestines.

Sensitization through the Placenta. That non-diffusible, colloidal materials can pass from mother to child has been well known for many years. The passive transmission of hypersusceptibility in guinea pigs was shown by Rosenau and Anderson (7) and by Otto (8) in their first studies on anaphylaxis. The transmission of diphtheria antitoxin from mother to child has been demonstrated by Polano (9) and von Groer and Kassowitz (10). Kuttner and Ratner (11) corroborated these investigations, titrating the antitoxic contents of maternal and cord blood, incidentally showing that in human beings no antitoxin is ever found in the colostrum or the breast milk. Similar observations of placental permeability have been made with tetanus antitoxin (12).

That bacterial toxins can be transmitted to the fetus is clear from the fact that the immunization of a mare with tetanus antitoxin produces antitoxic immunity in the foal. The transmission of foreign proteins as such through the placenta was first investigated by Ascoli (13) in 1902. He found that when considerable quantities of egg white or horse serum were injected into a pregnant rabbit, these could be detected by precipitin reactions in the fetal blood. These results have been confirmed by Holford (14). Irregularities in experiments of this kind when different species of animals are used find their explanation in the anatomical variations of placental structure throughout the animal kingdom. Grosser (15) has found that four main types of placentae exist, in which one, two, three, and four cell partitions respectively may intervene between maternal and fetal blood. The true adeciduate placenta found in pigs has four separating layers; the placenta in ruminants is composed of three. Man and rodents have only one connective tissue layer, and

according to the investigations of Ratner, Jackson, and Gruehl (16) such placentae are permeable to antibodies and to proteins. They found that horse serum sensitiveness could be transmitted to guinea pigs *in utero* both actively and passively. Milk and colostrum were negative in all experiments. There can be little doubt then that foreign proteins may enter the fetus *in utero*, and that both active and passive sensitization of the infant can take place before birth.

Direct proof of this in human cases has been reported by Ratner (17). By taking ante partum histories, he found, in certain children, protein sensitiveness to foods in which the mother had over-indulged during gestation. In such cases first contact with this particular food often gave rise to symptoms — an observation which considerably weakens the heredity conception of first contact cases.

Sensitization through the Intestinal Canal. The question of whether unaltered proteins can be absorbed through the intestinal canal has occupied investigators in anaphylaxis from the beginning, since this would be the most obvious method by which hypersensitiveness could be acquired. Uhlenhuth (18) showed that the feeding of egg white to rabbits resulted in the appearance of precipitins in the circulation. Ascoli (19, 20) found that in man egg white could be detected in the blood about two hours after the egg was eaten and soon after this in the urine. The same thing has been shown with chicken protein. We have found egg white in large amounts in the urine of monkeys a few hours after feeding. A few observers have failed to obtain positive results, but probably only because the methods applied were insufficiently delicate, since later observations have generally borne out the earlier ones. Van Alstyne (21) sensitized guinea pigs by the injection of blood serum and urine collected after feeding of large amounts of protein. Schloss and Worthen (22) investigated the passage of unaltered protein through the intestinal mucosa of children with gastro-enteric disease, and by immunological tests found food proteins in the urine. Anderson, Schloss, and Myers (23) found that cow's milk, egg albumen, sheep serum, and almond protein could pass through the intestines of marasmic children with considerable regularity and could often be detected in the blood and urine of normal children. In marasmic children the protein appeared promptly and remained present for considerable periods, whereas, in the normal children, passage through the intestine occurred only after weeks of feeding. Walzer (24) sensitized individuals by the passive, local method of Prausnitz and Küster with the serum of sensitive individuals. On the morning after the sensitization, before other food was taken, a measured amount of specific protein was fed. He used egg sensitive serum obtained from a child and fish sensitive serum obtained from two other patients. According to the degree of local sensitization,

a reaction appeared at the site of sensitization in the form of an erythema and slight edema within from a few moments to an hour after ingestion of the protein.

It is important to note that Walzer was using homologous serum, and when this is considered in connection with the negative results of others who used the Prausnitz-Küster technique with anti-egg rabbit serum on man, it suggests that the failures of such experiments were probably due to the heterologous nature of the serum.

It is plain, therefore, that human beings are constantly subjected to the possibility of intestinal sensitization and that were it not for the relative refractoriness of man and the hereditary indisposition of many individuals to become sensitized, a great many more human beings than are now affected would suffer from food idiosyncrasy.

Sensitization through the Respiratory Passages. Sensitization by inhalation is probable from clinical observation of the dust idiosyncrasies occupationally developed. Experimentally, respiratory sensitization has been demonstrated. Ratner, Jackson, and Gruehl (25) succeeded in producing respiratory anaphylaxis in guinea pigs by the inhalation of horse dander. This is of particular interest since horse dander, which produces a "typical" idiosyncrasy in man, thus sensitized one of the lower animals in a manner entirely analogous to that which occurs in the human disease. Ratner and Gruehl (26) also described the sensitization of guinea pigs by the inhalation of castor bean dust, producing in these animals a clinical picture similar to that occurring with the same substance in human beings. They thus demonstrated that animals can be sensitized to respiratory reactions with materials which act similarly in man.

The Influence of Heredity. Human hypersensitiveness depends for its development upon two factors: one, sensitization; the other, heredity. Whether or not similar differences in hereditary predispositions would be found to exist in animals were they sufficiently investigated, it is impossible to say, but we *do* know that individual animals of many species react with considerable differences of intensity of antibody formation to the same methods of immunization. The fact that individual horses are often unsuitable for antitoxin production is well known. Moreover, it must not be forgotten that most of those types of human hypersensitiveness for which the purely hereditary basis has been invoked are of the kind which appear only after years of exposure. And the life-span of the ordinary laboratory animals rarely exceeds several years.

In man there are infinite possibilities of exposure to the many antigens by which idiosyncrasies are elicited, and yet it is a fact that, of a large number of individuals exposed in the same manner, a limited number only develop sensitiveness. In factories in which sensitiveness to

quinine, nickel salts, etc., are acquired, relatively few of the workers are affected, and these after considerably varying periods of exposure. It cannot be questioned, therefore, that individual disposition has something to do with sensitization.

Cooke and Vander Veer (27) traced ancestral histories and discovered a considerable percentage with definite hereditary records of hypersensitiveness. They reported that of 504 cases 205 had a positive history on one side of the family, and 39 a positive history on both sides. Thus 48.4 per cent of the sensitive cases had a traceable history of inheritance as against 14.5 per cent of ancestral sensitiveness in normal individuals. Schloss (28) obtained satisfactory family histories in 80 cases. In 40 there was a history of allergy in parents, brothers, and sisters, and in 7 cases the parents were normal but there was a history of hypersensitiveness in the grandparents. In the remaining 33 cases, no family history of hypersensitiveness could be obtained, but of these 16 developed symptoms apparently on the first ingestion of the responsible food.

Cooke and Vander Veer believed that the inheritance follows Mendelian principles, though uncertain as to whether sensitiveness is dominant or recessive. It is probably quite impossible to determine genetic principles with any degree of certainty in view of the limited statistical material as yet available.

Important facts in appraising the significance of the hereditary factor, however, are the following: The child may be, but is not often born hypersensitive. In those with double inheritance, susceptibility develops in a considerable number of cases before the fifth year. With a single hereditary history, the most frequent age for the development of the condition is between ten and fifteen; and in those with a negative history, twenty to twenty-five years is the most frequent age for the development of symptoms. This suggests that whatever the hereditary factor may be, it does not usually develop until years after birth, during which it is impossible to exclude the possibility of numerous exposures to contact with antigen; and this, taken together with the demonstrated possibility of intestinal, respiratory, and intra-uterine sensitization, renders it impossible to exclude a specific sensitizing factor superadded upon a hereditary predisposition. Cooke and Vander Veer think that they have excluded this possibility by observing that idiosyncratic individuals were not more prone to develop horse serum sensitiveness after antitoxin administration than are normal individuals, but such reasoning is based on quantitative comparison of conditions in which many accidental factors play a role.

The second important fact in regard to the heredity of hypersensitiveness is the lack of specificity of the inheritance. Thus the child

may be sensitive to a substance quite different from that affecting the parents. The father may be idiosyncratic to fish, the mother to ragweed pollen, whereas the child may develop horse asthma — a state of affairs which should convince us that we are not dealing with a specific inheritance but, rather, with a tendency to become sensitive upon contact, in the same manner in which one rabbit may produce high antityphoid agglutinins after a course of treatment which, in another rabbit, may produce a relatively lower titer.

From all this it seems reasonably clear that heredity indicates merely a disposition to sensitization and that in the relatively few cases in which reactions occur upon first contact, there is probably a masked previous exposure, possibly intra-uterine.

Doerr, who was at first inclined in favor of a tentative separation of human idiosyncrasies from anaphylaxis, later modified these views. Coca at first supported a sharp separation chiefly on the basis of heredity. In a review of the subject (Tice's system, page 181*) he speaks of "the establishment of the influence of heredity as the sole determining factor in the causation of that condition" (hay fever). He previously, in a paper with Cooke and Flood on hay fever, stated that "hypersensitiveness is established spontaneously and never by immunological process." In the Tice article he wrote as follows: ". . . the clear demonstration of the operation of the factor of heredity in these two forms of allergy (hay fever and food idiosyncrasy) renders superfluous also the assumption of the anaphylactic nature of food idiosyncrasy"; and again, "we have associated the conditions we are discussing on the negative ground that an antigen-antibody mechanism had not been demonstrated — and the evidence at hand makes it appear, indeed, unlikely that such a mechanism is operative even in those allergies in which the exciting agents possess a protein nature." Cooke also took an uncompromising view which virtually implied the non-existence of immunological hypersensitiveness in man, who therefore possessed only a sort of immaculately conceived hypersensitiveness that, like his soul, distinguished him from the animal kingdom.

In later reviews, Coca, basing his change of opinion to a large extent on his own excellent researches, has receded somewhat from this position and retains in his newer classification only a small subdivision of so-called "normal hypersensitiveness" in which he places dermatitis venenata and serum sickness, the latter of which appears to us the most obviously anaphylactic of the human idiosyncrasies.

We have added this brief discussion of the controversy concerning the significance of the hereditary factors in order to do justice to views which, though we do not share them, are still held by some serious investigators.

The Passive Transmission of Human Hypersensitiveness. Since the first observation of the passive transfer of an idiosyncrasy by Ramirez, a number of other observations have been made which indicate that with proper technique it is occasionally possible to passively sensitize from man to man in cases in which transfer to animals has been impossible.

* For complete reference see bibliography, chap. XIV, p. 401, ref. no. 8.

It may well be that this phenomenon depends upon the necessity for species similarity of the immune serum to the protein of the animal which is to be sensitized.

Thus Frugoni (29) injected a large amount of serum of a woman who suffered from rabbit-hair asthma into a child, and a day later the child was specifically hypersensitive, and developed conjunctivitis and acute rhinitis upon contact with rabbits. Doerr (30) cites a number of analogous cases, accepting the possibility of such idiosyncratic passive sensitization without question. In most cases considerable amounts of the homologous serum were necessary, and as one would expect, recipients did not develop the specific localization found in the donors.

An important advance was made by Prausnitz and Küster (31) in 1921, when they succeeded in producing local hypersensitiveness in man by intradermal injections of small amounts of serum from individuals suffering from various forms of allergy. Küster was sensitive to fish, and on injecting a small amount of his serum intracutaneously into the forearm of a normal person and testing with fish protein some hours later, a definite local reaction was obtained. Apparently the "idiosyncratic" serum contained something analogous to an antibody but possibly not identical in every sense, since in this particular case as much as 2 cc. of Küster's serum failed to sensitize a guinea pig. DeBesche (32) successfully applied the Prausnitz-Küster technique in five cases of horse asthma. The antibody analogues which characterize such sera are spoken of as "reagins" by Coca, who has suggested the name "atopen" for the antigenic substances involved. It appears that the Prausnitz-Küster experiment is successful with the sera of all hay fever and asthma cases which give specific cutaneous reactions, but according to Coca and Grove only something over 80 per cent of normal skins are receptive. The local sensitiveness may last as long as three or four weeks. The "reagin" or antibody can be neutralized both in the test tube and in the tissues, but neither a precipitation reaction nor complement fixation can be shown in mixtures of the atopic antigen and the serum of the sensitive individuals. The P-K reaction has not been found successful in most drug idiosyncrasies or in bacterial allergy.

Relation of the Reagins to Other Antibodies. The Prausnitz-Küster discovery of passive homologous local sensitization in many of the "allergies" has definitely characterized reactions in which this technique is successful as based upon an immunological mechanism. After a latent period, as in passive anaphylaxis, a reaction can be elicited with a specific antigenic substance. So far the analogy with passive anaphylactic sensitization is obvious and the reagins, if not identical with the anaphylactic antibody, at least are closely analogous to antibodies.

The question arises, can these reagins be identified with antibodies or must they be regarded as Coca suggests as separate substances. Coca's original basis for such a separation was made because of the following points:

(1) The absence of demonstrable precipitin in atopic individuals
(2) The non-antigenic nature of many of the atopens
(3) The familial character of atopy
(4) The development of atopic sensitiveness without previous contact
(5) The absence of characteristic desensitization phenomena

Coca (33) admits that the non-antigenic nature of some atopens may be "tabled until the nature of drug idiosyncrasy is determined." That this has now been accomplished for most of the substances in question when this phrase was written has been made clear.

In regard to the failure of precipitation when reagin is added to its antigen this argument does not carry much weight, since we know that in order to precipitate there must be a reasonably high concentration of antibodies in a serum, much higher than is necessary for anaphylactic sensitization. Also, we know that the chemical and physical nature of an antigen may quite exclude the formation of a visible precipitate. As to the argument of the appearance of atopy without previous contact, this has lost much ground as a result of the information we have obtained regarding spontaneous sensitization of man, which has been discussed. The fact that it is impossible to transfer atopic sensitization with human blood to guinea pig skin or with precipitating serum from a rabbit to human skin are matters that may well depend upon the heterologous nature of the serum containing the antibodies.

Levine and Coca (34) and Coca and Kosakai (35) have reported that the mixture of atopen with the "reagin" serum deprives the latter of its sensitizing power, but that the activity of the atopen upon a sensitized site is not lessened in any appreciable degree by the presence of its related reagin. This is an important observation which must be carefully appraised. It may possibly be explained by slow dissociation in the skin not unlike the dissociation that must take place when toxin-antitoxin mixtures are introduced for immunization. As to the fact also observed by them, that repeated reactions can be obtained in the same sensitive site without increasing the concentration of atopen, a behavior quite different from that governing most anaphylactic reaction, this has been explained by Walzer and Grove (36) who found similar behavior in anaphylactic reactions with pollen-sensitive uteri in the Dale technique.

A further difference between the two types of hypersensitiveness emphasized by Coca concerns desensitization. He finds that specific treatment which successfully desensitizes individuals is not accompanied by a proportionate diminution of reagins in the blood. This, as Doerr (30) points out, may also be the case with anaphylactic animals. Local sensitiveness may have no relationship whatever to fluctuations of circulating antibodies. We must remember the attempts of Weil (37) to desensitize anaphylactic guinea pigs by the administration of large amounts of specific precipitating antiserum, in which he found that only excessive doses had any appreciable desensitizing effect.

Levine and Coca themselves have shown that therapeutic injections of pollen extract can appreciably increase the reagin contents of the blood — another important point which brings the reagin close to antibodies in general.

It is quite apparent, therefore, that although we cannot at the present time identify reagins with the well-known antibodies, yet there are so many

points of close similarity, that they must both be regarded as specific reaction bodies to a sensitizing antigenic substance. In this sense the "atopies" in which a known antigen is involved must be regarded as dependent upon an immunological mechanism, which in principle is not fundamentally different from anaphylaxis, except in so far as the anaphylactic responses of man are characteristic and different from those of other species.

It may well be that "reagins" are different from ordinary antibodies since some "antigens" determine only reagins in man, others only antibodies and others again determine the formation of both types. Even so, however, they must be, in both cases, regarded as specifically determined antisubstances and so accepted as variants of the same fundamental immunological mechanism — the reagin response being peculiar to man.

Desensitization in Human Idiosyncrasies. One of the most important similarities between human idiosyncrasies and anaphylaxis is the fact that specific treatment reduces the specific reaction capacity of the individual to the responsible material.

Occasional failure is not surprising in view of the quantitative relationships. We know that to desensitize a guinea pig completely in an ordinary anaphylactic experiment, it is necessary to administer doses which are near the threshold of danger; and in experiments with young goats carried out by us many years ago, we found that repeated anaphylactic shock can be produced in young animals if certain quantitative relations are observed. In man it is natural that the utmost care must be used not to give rise to systemic symptoms in hypersensitive individuals, and it is therefore not surprising that in many cases desensitization cannot be carried to the point of complete relief.

Nevertheless, the clinical results of Schloss, who has desensitized in food idiosyncrasies by careful feeding, and the general experience of clinicians in the treatment of hypersensitiveness by specific immunization methods have removed all doubt regarding the basic principle that such conditions are specifically influenced by immunization. These facts alone establish close analogy between human idiosyncrasies and protein anaphylaxis in animals.

Mackenzie and Baldwin (38) have demonstrated a type of local desensitization which can hardly be interpreted as anything but a temporary saturation of the sensitized tissues with the inciting substance. They found that by repeated applications to the same skin area, either by the scratch method or by intracutaneous injection of the responsible substance, reactivity could be locally abolished. This exhaustion was rapidly accomplished with horse serum, egg albumen, feather extracts, food products, and pollen extracts. The exhaustion was specific in so far as reactivity to one protein could be locally abolished with little or no diminution at that site to other proteins to which the individual was hypersensitive. The more highly reactive the substance, the more

rapidly the exhaustion was accomplished. Later, Mackenzie (39), applying these principles to hay fever, treated 38 patients, some by subcutaneous injections alone, some by local applications to the nasal mucous membrane alone, and others by a combination of both methods. He concludes that the reactivity of the nasal mucosa of hay fever patients may be markedly diminished by spraying the nose and throat with the pollen antigen; that patients given specific prophylactic treatment by this method reacted as favorably as those treated by subcutaneous injections, and that the best method was a combination of both (39). Caulfield (40) came to similar conclusions in analogous investigations upon 43 patients. He had previously found that specific cutaneous sensitization of a forearm might disappear over night after a local test.

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CHAPTER XIV

HYPERSENSITIVENESS OF MAN, *Continued*

A. Serum Sickness

BEFORE we knew anything about anaphylaxis, experiments like those of Bertin (1) and of Johanessen (2) had shown that the injection of horse serum in antitoxin administration produced a train of abnormal phenomena which were referable to the foreign protein injected and had nothing to do with the antitoxic contents. References to this condition were made in medical literature soon after the beginning of the therapeutic use of horse serum, but no analytical study was carried out until the work of von Pirquet and Schick appeared in 1905 (3).

The term "serum sickness" has now come to possess a technical meaning indicating the consequences of foreign serum injection into man.

In the technical sense the term "serum sickness" signifies a train of symptoms which frequently follow a week or longer after the injection into a human being of foreign protein. Since, in the overwhelming majority of procedures of this kind, the inciting substance is a therapeutic serum, usually horse serum, the condition has been most extensively studied in this connection. The symptoms to be described occur not only in individuals previously sensitized to horse serum but are observed in a considerable percentage of persons after a first injection, even when it is reasonably certain that there has been no sensitizing contact. It is this point which has led to doubt in the minds of some observers concerning the true anaphylactic nature of the condition. This question will be discussed in detail below.

In first injection cases, the incubation time is usually from six to ten days. The symptoms vary, but fall into a characteristic group which sufficiently defines the condition, though one or another of these symptoms may be lacking at times. Skin eruptions occur in from 69.4 to 81.9 per cent of the 600 antitoxin cases studied by Rolleston (4). Of the eruptions, urticaria is the most common and may begin near the point of inoculation and spread over the body. The next most common form of skin manifestation is a diffuse erythema not unlike that of scarlatina.

Edema of the face is not uncommon. Edema of other parts of the body such as the ankles may occur. Longcope (5) mentions a case of one

patient in whom a transient hemiplegia was attributed to edema of the meninges.

Joint pains often accompany the rash and may offer considerable diagnostic difficulty. We have seen cases following tetanus antitoxin in which there was doubt as to whether the stiffness of the joints was serum sickness or due to early tetanus.

The temperature may often rise slightly and with it a general feeling of illness, headache, and weakness appear. There may be some albuminuria and, occasionally, oliguria. Nausea and vomiting are relatively rare. A common symptom is enlargement of the lymph nodes, particularly in the area of the serum injection. Increased leucocytosis may occur at first followed by leucopenia and relative lymphocytosis.

The symptoms may subside after one or two days, but often last a week and more and, with very large serum injections, relapses have been observed. Axenow, whom we quote from Longcope (6), speaks of ten-day intervals between such relapses.

An infrequent but important symptom complex in serum sickness is one which involves a uniform type of paralysis of the upper extremities, usually confined to the right side and frequently involving only muscles innervated by the fifth cervical root. The paralysis follows root distribution, develops rapidly during or just after the height of serum sickness, and is preceded by excruciating pain. The prognosis is uniformly favorable for complete restoration of function. This exceptional symptom complex is described because it has given rise to much diagnostic difficulty and anxiety. Dr. James B. Ayer (7) has discussed 40 cases from the literature and 5 of his own, and has come to the conclusion that the condition is undoubtedly a part of the serum sickness complex. Since a good many of these cases occurred after injection of antitetanus serum, the suggestion has been made that there is a peculiarity in connection with this particular antitoxin which can be held responsible for the condition. The location of the paralysis has no regular relation to the site of injection. Of 24 of the cases studied by Ayer, in only 8 did paralysis occur in the limb injected, whereas in 16 the paralysis was on the opposite side. An explanation for this symptom complex is entirely lacking. It is mentioned, however, because of its considerable clinical interest and because of the necessity for the immunological study of such cases.

Von Pirquet and Schick in their first description differentiated between symptoms after a first injection and those following repeated administrations. As a general rule, a second injection is more rapidly followed by symptoms than a primary injection, *i.e.*, after a shorter incubation. After primary injection the average time before the appearance of symptoms is from six to ten days. In individual instances, such as the case of Daut, which we quote from Coca (8), the incubation period after a primary injection was only three hours; and Coca mentions that in Hartung's series over 20 per cent had symptoms within the first three

days. These may well have been cases of masked (intestinal, etc.) sensitization. After second injection the average incubation time is considerably shortened, averaging about three to five days, often being shorter than this. Such shortened incubations are spoken of by von Pirquet and Schick as the "beschleunigte" or accelerated reactions. Repeated injections may give rise to elimination of any incubation time in what von Pirquet and Schick speak of as the "immediate" reaction. Within certain limits speed of onset depends upon the intervals between consecutive administrations. In von Pirquet and Schick's studies, when the interval was twenty-one days or less, the immediate reaction alone was noticed; when it was between two and three months, both immediate and accelerated reactions were present; and when the interval was still longer, the accelerated reaction alone was observed. Currie (9) confirmed the observations of von Pirquet and Schick, and while, of course, his intervals are not identical with theirs, the general principle that the nature of the reaction depends upon the interval between injections is upheld. Goodall (10) agrees with these conclusions, but adds that if one reinjects within a short period after the first administration, say a week or less, a temporary refractory condition is produced, but eventual sensitization supervenes. These facts clearly suggest analogy with animal anaphylaxis and the relation of symptoms to interval correspond fairly well with the conditions one would expect at periods of fluctuating degrees of sensitization. Longcope states that the period after the first injection at which the immediate reaction is most likely to occur lies between the thirty-fifth and eightieth days after a preceding injection, and, at such times, is apt to occur in about 60 per cent of the cases if sufficient amounts of serum are injected. The immediate redness, swelling, and edema with localized lymphatic enlargement which occur in such cases are, in appearance, strikingly similar to the Arthus phenomenon in rabbits.

It is obvious that the *delayed*, *accelerated*, and *immediate* reactions shade into each other both in regard to time and severity, and that the influence exerted upon the condition by preceding injections indicates that something closely analogous to sensitization in animals is operative.

That sensitization to horse serum in human beings is experimentally possible has been brought out by Hooker (11), whose work refutes the assertion of Cooke and Vander Veer (12), that artificial sensitization is not easy to accomplish in man. Hooker studied individuals who had been immunized to diphtheria with toxin-antitoxin injections in which the amount of horse serum injected is extremely small. He found that intradermal tests with horse serum carried out six months after toxin-antitoxin injections in previously negative individuals became either

immediately positive or developed a delayed positive reaction in 62 out of 96 individuals. The positives who had previously been treated with horse serum developed, from an originally delayed reaction, an immediate type of response after the prophylactic treatment. Cowie has reported a case of positive skin reaction induced by a previous injection of 0.1 mg. of horse serum. Longcope and Rackemann (13) also have noticed that horse serum both in large and small amounts is followed, sooner or later, by the development of skin hypersensitiveness.

The anaphylactic sensitizing effect of previous serum injections is also quite evident from observations such as those made by Darling (14), who was obliged to inject antiplague serum prophylactically into 17 people. The amounts administered were 10 cc. Only three of these patients reacted, and these three were the only ones who had had previous injections of horse serum in the form of diphtheria antitoxin. Although these sensitizing injections had all been made as long as six years before, two of the subjects showed accelerated reactions and one an immediate reaction. Darling's experience, as well as other facts which we have cited, suggests that a good deal of the confusion concerning this question of sensitization has arisen as a consequence of disregard of quantitative relations. The large statistical material on which most conclusions are based includes a great many cases in which amounts much larger than 10 cc. were administered. In the case of small amounts, differences between previously sensitized and normal people are likely to be much more noticeable than in cases in which massive doses are injected. In Weaver's studies, only about 10 per cent of the individuals receiving 10 cc. or less develop serum sickness, and in these the condition is usually mild. The principle of von Pirquet, as analyzed by Longcope and others, by which serum sickness resulting from first injection is taken to depend upon the persistence of antigen in the circulation after the formation of antibodies on the cells has begun, is most easily determined under circumstances in which, after injection of considerable amounts of serum, the antigen which must be eliminated is correspondingly large. The observation that serum up to 10 cc. gave rapid and severe serum disease only in the members of a controlled group who had been previously injected with the same antigen lends additional and strong support to the conception of serum sickness as an obvious variety of true anaphylaxis.

The results of Hooker, together with the other evidence cited, conclusively demonstrate that horse serum injections even in minute amounts may actively sensitize human beings. The question of the effect of such sensitization upon therapeutic serum administration was discussed, after Hooker's communication, by Park (15), who concluded that there need be no especial fear of reinjecting antitoxin or serum, in

spite of evidence of sensitization, provided suitable precautions were observed.

Acute dangerous and fatal shock is fortunately a rare occurrence. Park (16) has reported that of 105,000 subcutaneous injections given between 1895 and 1913 only two deaths followed such injections and both of these followed the primary injection rather than later ones. In a third case there was a temporary collapse during a second injection given intravenously. In one of the fatal cases autopsy showed status lymphaticus. Ustvedt, whom we quote from Doerr (17), saw only one such case in 18,000 prophylactic subcutaneous injections of horse serum. When the serum is given intravenously in considerable quantities, the number of acute cases may be more frequent, although no extensive statistics have been made on this problem, but certainly with ordinary precautions as they are observed in pneumococcus therapy for instance, there have been extraordinarily few acute shock deaths. Park (18, 19), covering 350,000 injections, found that one acute death occurred for every 70,000 people injected with horse serum. In a later analysis Park (20) estimates that one in 20,000 serum injections is followed by alarming symptoms and one in 50,000 is fatal.

In 1924 Lamson (21) of our laboratory searched the literature for cases of sudden death following injections of foreign protein. From the beginning of diphtheria antitoxin therapy in 1894 up to 1910, he found reports of a total of 41 cases of sudden death, some of which were undoubtedly duplications, and he calls attention to the fact that in this series there were a few which were certainly not immunologically related to the serum injections. From 1910 to 1924, a period during which there was an enormous increase in serum therapy as compared with the preceding years, he found only seventeen authenticated cases. In an analysis of all the cases he found that 34 per cent gave a history of asthma or hay fever and several of these were cases of horse asthma. In eight of the fatal cases there was a history of previous injection of foreign protein.

The extraordinary sensitiveness to horse serum of certain "horse asthmatics" imposes the greatest caution. Boughton (22) tells of a patient in whom death followed the injection of 0.06 cc. intravenously. Such cases are fortunately rare, but when serum is to be given to a horse asthmatic all possible precautions, *i.e.*, preliminary desensitization with minute doses, must be carried out. This problem will be separately dealt with.

In looking over the various studies of acute cases one has the impression that while there are unquestionable cases of acute death which may be set down to allergic reactions, a certain percentage of the fatalities must be attributed to those obscure forms of death which occasionally follow the administration of ether or puncture of the pleura. Investigations by E. F. Müller (23, 24, 25) promise to throw a certain amount of

light upon these, since he has shown that the intracutaneous injections of minute amounts of sterile water or air may, in certain individuals, arouse powerful sympathetic vasomotor reflexes.

It is to be expected that danger is greater with intravenous infections than with the subcutaneous method, because of the greater speed with which the antigen comes in contact with the sensitized tissues, yet even intravenous administration has rarely led to fatal accident. Park mentions that in 300 consecutive cases of intravenous injections there was not a single severe reaction, and the almost habitual use of the intravenous route for antipneumococcus serum in enormous numbers of cases with very few accidents has demonstrated the essential safety of this method if proper precautions are taken.

Intraspinous inoculation has been followed by acute symptoms in a number of cases, Auer * reporting three cases from the literature in 1912.

When such acute shock occurs, it has many of the characteristics of anaphylactic reactions in animals, such as dyspnea, disturbances of the pulse, drop of blood pressure, rise of temperature, abdominal pains, and sometimes loss of consciousness. Such cases are not always fatal, and may fully recover within a few hours. Frequency depends to a great extent upon the amounts administered. Weaver (29) in a study of 801 reported cases found that only about 10 per cent developed serum sickness when amounts lower than 10 cc. were given, and in these the resulting disease was usually mild. When 90 cc. or over were administered, the condition developed in 75 per cent of the cases and was often more severe.

The unwillingness of some students of *serum sickness* to accept the condition as an example of human anaphylaxis has largely rested upon the fact that the symptoms may occur, without preceding sensitization, after the first injection of serum. The views of von Pirquet and Schick were based upon the idea that, as the antigen is gradually brought into contact with the cells of the body, antibody begins to form and that antibody formation precedes the complete elimination of the antigen. That this is actually the case was shown by the experiments of von Dungern and others, who demonstrated that antigen and antibodies may be present in the circulation of an animal at one and the same time, especially after the injections of considerable amounts. The newly formed antibodies may then react with the antigen residue still present. In support of this idea, von Dungern cites the appearance of precipitins in the blood of a rabbit injected with horse serum, finding that precipitins occurred as early as the eighth or ninth day at a time when horse serum

* The cases reported by Auer were published by Hutinel (26), Grysez and Dupuich (27), and Archard and Flandin (28).

could still be detected in the same blood. The matter has been extensively studied by Longcope and Rackemann (13) and by Longcope and Mackenzie (30). Longcope and Rackemann found that the blood serum of most patients who suffer from an attack of serum disease following injection of horse serum shows precipitin for horse serum, and that such serum could passively transfer the anaphylactic state to guinea pigs, thus bearing out the work of Hamburger and Moro (31) and of Wells. They found, also, that these antibodies could not be demonstrated in the blood serum of patients who did not later present symptoms of serum sickness, and that the appearance of the antibodies shortly preceded recovery from the disease. Subsequent studies by Longcope and his collaborators on 19 cases led to the conclusion that there is a definite relationship between antibody formation and the severity of the symptoms and that severe reactions develop chiefly and "perhaps exclusively" in those individuals who develop a high concentration of precipitin in the circulation. They found that in such patients the horse serum is apt to disappear from the circulation soon after a large amount of precipitin appears, but that in many cases the antigen and antibody can be shown to coexist for variable periods. Some individuals seem to be relatively insusceptible to serum reaction, and these are the cases in which little or no precipitin is formed. The tables of Longcope and Mackenzie show a number of cases in which failure of the development of serum disease was accompanied by failure of antibody production, and also a considerable number of cases in which a number of symptoms attributable to serum disease preceded the observation of any antibodies in the blood. Indeed, in one case symptoms appeared 24 hours after injection, in several cases after two days, and in a number of cases between the fourth and seventh day. Such observations offer no theoretical difficulties since one would expect cellular hypersusceptibility to precede the discharge of antibodies into the circulation.

Thus, in analogy with anaphylaxis, serum disease develops after the injection of an antigenic substance; when it comes on after first injection the incubation period may be, but is rarely, less than 5 days, and is usually longer than this; and finally as von Pirquet, Goodall (10), and Currie (9) have shown, second and subsequent injections give rise to a shortening of the incubation period.

The chief objections that have been made to this anaphylactic conception of serum sickness have been based upon the fact that reactions occur in individuals who have never had horse serum before, that there is doubt as to whether human beings can be actively sensitized at all by the usual methods, that there is no regularity of relationship between the presence of precipitins and serum disease, and that desensitization

in serum sickness is difficult. The foregoing paragraphs have answered most of these objections. The facts may be summarized as follows:

Serum sickness is a specific reaction to an antigenic substance. That human beings can be artificially sensitized to reaction with this antigen is apparent from the shorter incubation times and the greater intensity of symptoms which follow second and subsequent injections, as contrasted with the first injection. Experiments of Hooker, of Darling, and of others have demonstrated the possibility of human active sensitization with horse serum. The occasional but rare immediate reactions observed at first injection have lost weight in view of the demonstration of intrauterine and intestinal sensitization. The recurrence of symptoms in serum sickness without reinjection after latent intervals of a week or ten days may, as Doerr points out, be due to the presence of a number of separate antigens in horse serum. Hooker (32) has shown that at least three separate antigenic fractions of horse serum may cause reaction in man and that these reactions occur at varying times after intracutaneous injection into sensitive individuals, the first fraction giving a reaction after 20 minutes, the second after 5 hours, and the third after 12 hours. It may well be that individuals actively sensitized with horse serum develop precipitins against the individual serum fractions at different times.

As concerns the presence of precipitins in the blood of individuals with serum sickness, the investigations of Wells and of Longcope have satisfactorily demonstrated that antibody formation occurs in close parallelism with symptoms and that with the blood of individuals with the characteristic disease active and passive sensitization of guinea pigs can be accomplished.

It is not even necessary that sensitizing serum should actually be capable of precipitating the antigen. Cells may be sensitive when antibody has disappeared from the blood and antibodies must be concentrated considerably in a serum before they will lead to visible precipitation. Feeble antibody contents of a serum may still be capable of sensitizing an animal, as detectable by the anaphylactic test; or of sensitizing an antigen *in vitro*, as can be proved by complement fixation, without being sufficiently concentrated to cause precipitate formation.

It has been suggested that the heterophile antigens present in horse serum might play a role in the causation of serum sickness. This question has been studied in detail by Powell, Jamieson, and Kempf (33), who have come to the conclusion that there is no connection between serum sickness and reactions between heterophile antigen and antibody. Theoretically, it is not impossible that a certain amount of reaction might result when horse serum is injected into man, but the phenomena of serum sickness as it occurs under ordinary circumstances are quite sufficiently accounted for by the mechanism which has been described.

Desensitization in Serum Sickness. Although the danger of fatal accident in the administration of antitoxic sera is not great, yet the occasional fatalities and the acknowledged danger in asthmatics makes it necessary, not only to apply some test for sensitiveness but also to attempt desensitization in susceptible patients in whom serum is therapeutically indicated.

One of these methods is the "Skin Test" described in the following paragraphs on precautions to be observed in the administration of serum.

In addition to the skin test, sensitiveness of a given individual may be determined by the instillation of the antigen — in this case, horse serum — into the conjunctival sac. Spicer (34) has made a comparative study of the two tests and believes that the ophthalmic reaction gives a more practically useful indication of whether or not serum can be safely given. The ophthalmic test is not as sensitive as the skin reaction, and in Spicer's series a drop of undiluted normal horse serum gave no serious reactions. The test was positive only with concentrations higher than 1–10. The patients who gave positive ophthalmic tests gave the strongest skin tests and the most marked serum reactions after therapeutic injections of antitoxin. Reactions appear from ten to fifteen minutes after the application of the serum and clear up within one-half to two hours. All patients who gave ophthalmic tests were positive intradermally, and Spicer claims that patients who gave negative ophthalmic tests with the lower dilutions of horse serum — that is, dilutions of 1–2 to 1–10 — never reacted strongly to antitoxin. In her opinion the intradermal test is so delicate that it may induce positive tests in patients who subsequently show no serious reactions to serum injection.

Precautions to Be Observed in the Administration of Serums. Reactions may occur in spite of every precaution, but their frequency may be diminished by the observance of certain measures.

History. Before the injection of serum, all patients should be questioned about their having had asthma, hay fever, eczema, urticaria, angioneurotic edema, or hypersensitiveness to horse dander or horse serum. Serum should not be given *intravenously* to those with such a history.

They should also be questioned as to whether they have previously had horse serum (diphtheria, tetanus, or other antitoxin, antimeningococcic or antipneumococcic serum, diphtheria toxin-antitoxin mixture, etc.). If the history is positive, the administration of serum should depend upon the outcome of the tests described below.

A reliable history is essential. Young patients, or those who are very ill, may be unable to give a proper history, in which case it must be obtained from other members of the family.

It is always important to impress upon the patient, or those responsible for him, that they must remember the fact that serum has been given and the time at which it is administered, for report to the physician should another serum injection ever be called for in the future. At the same time, the difference between "vaccine" and "serum" must always be made thoroughly clear, since even physicians not infrequently confuse these terms.

Tests for Hypersensitivity. At least one of the following tests should be used in all cases where serum is to be administered intravenously. A test is also desirable when serum is to be given by any other route.

1. *Ophthalmic Test.* This is done by putting one drop of serum (undiluted or diluted 1–10) into the conjunctival sac of one eye. A positive reaction is indicated by itching, watering, and a diffuse reddening of the eye within 30 minutes. An extreme reaction may be controlled by the instillation of a few drops of epinephrine (adrenalin), 1–1000 dilution.

This test is of no value in young children, for they may wash out the serum by crying.

2. *Skin Test.* Have at hand, ready for use, a syringe containing 1 cc. epinephrine (adrenalin), 1–1000 dilution. A further supply of epinephrine should be within reach.

0.1 cc. of physiological salt solution is injected as a control *into* (not under) the skin of the anterior surface of one forearm, and 0.1 cc. of a 1–100 dilution of normal horse serum is similarly injected in the other forearm. The dose used for intracutaneous test is often even larger than the one just mentioned, some observers using as much as 0.1 cc. of a 1–10 dilution. In cases in which the history is one which indicates probable hypersensitiveness, it is best to begin with the smaller dose. The injected fluids each produce a small white wheal at the site of injection, both of which disappear within a few minutes if the test is negative. (The wheal produced by the serum may not disappear quite so quickly as that due to the saline.)

In sensitive patients the skin elevation at the site of the serum injection rapidly enlarges within 5 to 20 minutes and becomes urticarial in appearance with a surrounding zone of erythema. The size of the wheal is perhaps a rough indication of the degree of sensitivity. In strongly positive tests, pseudopodial extensions of the central wheal are noted. Positive skin tests ordinarily subside within an hour or two.

Interpretation of Tests: Although the tests described do not always give an accurate indication of hypersensitivity, these tests, together with the patient's history, constitute our only means of determining it. The following rules are suggested as a guide to treatment.

1. Do not give serum *intravenously* to patients with: (1) a positive

history of asthma, hay fever, etc.; or (2) a positive or questionably positive ophthalmic test, unless the therapeutic indications for serum are urgent as in malignant diphtheria, meningitis, etc.

2. Do not give serum *intravenously* to patients with positive skin test, unless they are first "desensitized" by the method outlined below or a similar one.

3. Serum may be given by any route in patients with negative histories and tests. If given intravenously, the serum should be warmed to body temperature and given slowly (1 cc. per minute). In giving the first intravenous dose it is desirable to wait 15 to 30 minutes after 1 cc. has been given before further amounts are injected.

4. Patients should be kept under observation for three quarters of an hour after any administration of serum.

Desensitization: Hypersensitive persons may be partially desensitized by the repeated administration of very small doses of serum. The method is not uniformly successful but should not be omitted when history or tests indicate sensitiveness.

Epinephrine (adrenalin), 1–1000 dilution, must be at hand in a syringe and ready for use should a reaction occur.

Administration of serum is begun with subcutaneous injections of dilutions of normal horse serum or of the serum to be used for treatment. The initial dose is 0.005 cc. (0.5 cc. of a 1–100 dilution). If no reaction occurs within 15 minutes, the dose may be doubled; and so long as no reaction intervenes further doses are given at 15–20 minute intervals, using twice the amount given at the preceding dose, until a dose of not less than 1 cc. of *undiluted* serum has been given subcutaneously without reaction. These directions should be taken to represent only an example of average procedure. No precise rules can be laid down for all cases. The responsible physician should be familiar with the possible reactions that may occur and should adjust quantities and time intervals according to judgment in the individual case.

Further treatment depends upon the kind of serum to be given and the route of administration. For subcutaneous, intramuscular, or intraspinal administration, the full dose may be given 30 minutes after the last subcutaneous dose. For intravenous treatment, more caution is required. The first intravenous dose following the last subcutaneous one of at least 1 cc. of undiluted serum should not exceed 1 cc. of serum diluted 1–10. This may be followed 20 to 30 minutes later by twice as much serum, and further doses, doubling in amount each time, may be given at similar intervals. Inject the serum slowly — not faster than 1 cc. per minute.

If any injection is followed by edema, urticaria, or respiratory distress, the symptoms may be relieved by epinephrine. If more serum is given,

the next dose of serum should not be larger than the previous dose (the one causing the reaction) and should not be given until at least 30 minutes later. If this also causes reaction, no further serum should be given intravenously.

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CHAPTER XV

HYPERSENSITIVENESS OF MAN, *Continued*

Gastro-intestinal, Respiratory, and Drug Idiosyncrasies

The Gastro-intestinal Type of Human Hypersensitiveness (*Food Idiosyncrasies*). Hypersensitiveness to the ingestion of various kinds of food — milk, meat, eggs, sea food, strawberries, etc. — is a well-recognized clinical entity. In preliminary discussions we have indicated how such sensitiveness can be acquired. The child may become actively sensitized *in utero*, as Ratner (1) has shown, because of the mother's over-indulgence in certain protein foods during the ante partum period, or the child may become sensitized after birth by ingestion. Schloss and his collaborators (2) have proved that proteins will pass through the intestinal wall not only in marasmic children, but in normal children as well. It is thus unjustified, even when a reaction results from the first known contact with a food, to assume that the condition is congenital.

The localization in the intestinal canal in such cases may be interpreted either as a particularly severe sensitiveness of the gastro-intestinal walls because ingestion sensitizes the tissues of concentrated contact more intensely than the rest of the body, or it may mean simply that, the body as a whole being sensitive, the antigen ingested comes into most concentrated contact with the gastro-intestinal apparatus, which therefore reacts most actively.

Intestinal anaphylactic symptoms have been frequently described in animals. As early as 1911, Weichhardt and Schittenhelm (3) described local reactions in the gut in anaphylactic dogs. Manwaring and his collaborators (4) confirmed Weichhardt's observations and found edema of the intestinal mucous membrane followed by epithelial desquamation and sometimes by superficial necrosis during the later stages. They attributed the lesions to prolonged contraction of the intestinal musculature with consequent stasis of the circulation in the mucous membrane.

Sometimes the reactions are confined to the mouth and tongue, and minute amounts lead to edema, swelling, and difficulties of swallowing. In other cases the entire gastro-intestinal canal may be involved. Immediately after ingestion there may be abdominal discomfort, nausea, and a desire to vomit. Wiedemann (5, 5 a), who has observed the reactions following the feeding of the responsible material to patients

together with barium, has found that the conditions are much as described in dogs by Manwaring — namely, powerful contractions of the pylorus, increased peristalsis, and later severe contractions in the lower bowel. In the course of several hours there may be colic and diarrhea. Systemic symptoms, weakness, rapid pulse, and low blood pressure, occur in the severe cases, and many attacks are accompanied by skin manifestations such as urticaria, which may begin within an hour after ingestion. Angioneurotic edema of the face is not uncommon.

The most severe cases are those in which the taking of food is followed by so rapid and violent a reaction that spitting and vomiting occur within a few minutes and there is a rapid development of swelling of lips, tongue, and mouth. Such a case has been described by Schloss in a child. Although the attacks follow ingestion of the responsible substance, the manifestations may consist in some cases of only the systemic symptoms of protein hypersensitiveness without involvement of the intestine. An urticarial eruption may be the only symptom. In the more or less protracted gastro-intestinal allergies which either underlie or accompany marasmus in children, eczema is a common symptom. Schloss (6) has traced many cases of eczema and of erythema to hypersensitiveness, improving or eliminating the condition by omission of the responsible foodstuffs. Similar observations have been made by O'Keefe (7), who found that many exclusively breast-fed infants showed positive skin reactions to egg protein or to cow's milk. He attributed this to sensitization through the mother's milk, basing his belief on Shannon's (8) demonstration of food proteins in human breast milk. O'Keefe obtained improvement in the children by excluding the offending protein from the mother's diet.

Schloss, Bruck (9), and many others have found that in most food idiosyncrasies specific skin reactivity goes parallel with the hypersensitiveness, but Schloss has noticed that occasionally the skin reaction does not develop. There are, in other words, rare cases in which the distribution of the sensitiveness does not include the skin.

The differences in local distribution of response which may prevail under identical circumstances of sensitization are illustrated in twins studied by Ratner (10). Both gave skin reactions to lactalbumin and both were sensitive to cow's milk, but one of them developed urticaria, the other vomiting and diarrhea.

Schloss (11) has also succeeded in simulating the human condition in guinea pigs. By feeding guinea pigs considerable amounts of egg white following a short period of starvation, he sensitized them so that 13 days after sensitization they responded with anaphylactic symptoms to injected egg white and, in some of these animals, diarrhea and watery stools followed the shock injection. He was also able to convey specific

hypersusceptibility to guinea pigs with the serum of food-idiosyncratic individuals. With other food idiosyncrasies only the homologous local sensitization of Prausnitz and Küster has been successful. The fact that Coca and Grove (12) failed to transfer egg sensitiveness with an egg precipitating rabbit serum to the skin of man does not invalidate these observations, since the rabbit serum was heterologous.

Desensitization in the food idiosyncrasies is usually a difficult matter. In three of Schloss's cases he completely desensitized against ovomucoid, starting with 0.0001 mg. in saline solution and injecting at intervals of 5 days, doubling the injection at each dose. In this way he found, with the development of immunity, the fading of the cutaneous reaction. Gradually increased feedings of the responsible protein have also had desensitizing effects. Initial doses were given in capsule form in amounts of 2 to 5 mg., diluted in milk sugar or starch. One infant, one year of age, was so sensitive to milk that swelling of the tongue and lips resulted from one drop of milk diluted with water. Schloss started in this case with the administration of a 20th of a drop 3 times a day.

If we survey our knowledge of the gastro-intestinal types of human hypersensitiveness, limiting our discussion to those due to antigenic materials such as the proteins, it seems that there is very little that can be opposed to the conception that these food susceptibilities are nothing more or less than a form of human anaphylaxis. The inciting substances are antigens; sensitization can be demonstrated as taking place through placenta or bowel; antibodies are involved, both as shown by their development in the blood of protein-fed marasmic children and by Schloss's guinea pig transfers; skin sensitiveness develops and desensitization is successful within the limits with which this is possible in man. Antibodies which transmit the condition to guinea pigs have been found and at the same time the Prausnitz-Küster reaction has been successful — facts which point to overlapping of the two methods of passive transfer for reasons not yet clear.

The Respiratory Forms of Human Idiosyncrasies. Hypersensitiveness that is characterized by symptoms centering in the respiratory organs is common. Most important is the condition known as hay fever, which is caused by various forms of pollen, chiefly the ragweeds. Similar conditions can be caused by a great many forms of dust and emanations from both animals and plants. Common among these are the dandruff and hair of horses, cats, guinea pigs, rabbits, and other animals, orris powder, certain forms of house dust, feathers, vegetable meals, and rusts and industrial dusts, especially those of chemical factories. The last are considered under drug idiosyncrasies.

The symptoms of such respiratory hypersensitiveness may be limited to attacks of spasmodic rhinitis and conjunctivitis with few if any

systemic symptoms. They may, however, develop into severe attacks of asthma.

Antigens in Respiratory Idiosyncrasies. It is important in determining the relationship of these idiosyncrasies to true anaphylaxis to know whether the substances which incite them have the characteristics of true antigens.

Cook, Flood, and Coca (13) succeeded in actively sensitizing guinea pigs to extracts of horse dander. The same thing was done by Friedberger and Kamio (14). Walker (15) obtained positive complement fixation reactions with a horse dander extract antigen and the serum of 3 to 11 patients sensitive to horse dander.

Longcope, O'Brien, and Perlzweig (16) succeeded in highly sensitizing guinea pigs to extracts of horse dander and desensitizing them by repeated treatments. The problem arises, How is the horse dander antigen related to horse serum? Longcope and his collaborators found that horse dander was specific and did not cross react with horse serum. Forster (17) observed slight cross reaction both in precipitation and in anaphylactic experiments. The dander antigen responsible in horse asthma is therefore quite distinct from the serum protein. That the species specific serum protein is antigenically quite distinct from similarly species specific cell proteins is a point which, in immunology generally, and especially in cytotoxin and heterophile antigen investigations, possesses considerable importance.

Grove and Coca (18) found that trypsin digestion and dialysis of dander antigen and of pollen causes a diminution of atopen activity corresponding with the loss of protein by digestion.

Can Respiratory Hypersensitiveness Be Experimentally Produced? Respiratory types of hypersensitiveness have been experimentally produced in animals. Friedberger (19) obtained local pulmonary symptoms by subjecting horse-serum sensitive guinea pigs to sprays of horse serum. Busson (20) successfully sensitized guinea pigs by inhalation of ox serum. Nasal instillation seemed to give the best results to Sewall and Powell (21, 22), who produced a form of bronchial asthma by repeated nasal administration of horse serum. Work of similar significance has been carried out by Busson and Ogata (23), Sternberg (24), Jones (25), and others. Ratner, Jackson, and Gruehl (26), using dried horse dander, sensitized guinea pigs by inhalation, produced respiratory symptoms and general anaphylactic death by subsequent inhalations, and showed that epinephrine alleviated the symptoms in such asthmatic guinea pigs just as it relieves the conditions in man. One of the most interesting parallelisms between human respiratory hypersensitiveness and an experimental condition in animals is the castor bean dust observation reported by Ratner and Gruehl (27). Figley and Elrod (28)

had noticed that individuals living in the vicinity of castor oil factories acquired asthma from the inhalation of "pomace", or castor bean dust, an assumption which was supported by positive skin reactions. Ratner and Gruehl sensitized guinea pigs by inhalation of the dust and produced respiratory anaphylaxis in these animals. They also found that immunity to the dust could be produced by repeated inhalation.

Passive Sensitization in the Respiratory Idiosyncrasies. Ramirez (29) reported passive transfer of hypersensitiveness to horse dander by the transfusion of blood from a hypersensitive to a normal man.

Horse dander hypersensitiveness has been repeatedly transferred by the Prausnitz-Küster method by DeBesche (30), by Coca, and by others. DeBesche reported that he was able to transfer hypersensitiveness to horse serum from man to guinea pigs with the blood of 3 out of 8 horse asthmatic patients. These experiments are questionable in view of their contradiction of the specificities of horse dander and horse serum antigens.

Desensitization in the Idiosyncrasies. There is evidence that the specific treatment of respiratory hypersensitiveness, particularly hay fever, can be favorably influenced by treatment with the responsible antigens. This method, based upon anaphylactic desensitization, was further developed by Noon (31), by Clowes (32), by Cooke and Vander Veer (33), and by Freeman (34, 34 a). Patients who have received graded subcutaneous injections (for details see Rackemann (35)) are often improved, temporarily relieved, or actually cured for periods of one or two years. There is to this extent complete analogy between anaphylactic desensitization and the specific relief of an idiosyncrasy. Differences between the two phenomena of desensitization cannot be ignored in discussions which compare the mechanisms of the two conditions. One important discrepancy has been reported by Cooke to the effect that while individuals who have been relieved of their clinical symptoms in consequence of hay fever treatment have not lost their skin reactiveness nor the sensitiveness of their conjunctivae to the extract. Coca and others (36) who have analyzed the relationship of reagin and antigen or "atopen" in the Prausnitz-Küster experiment have found that the reagin can be neutralized both in the test tube and in the tissues, the mixture of atopen and reagin failing to sensitize. When the atopen was brought into relation with reagins that were attached to certain fixed tissues, *i.e.*, sites of passive sensitization, there was desensitization of the site. On the other hand, Levine and Coca (37) showed that in similar mixtures of reagin and atopen the atopen remained active and was not diminished in its action upon sensitized tissues.

Desensitization by local contact has been studied by Mackenzie and Baldwin (38). They showed that cutaneous hypersensitiveness can be

locally abolished by repeated application of the responsible substances to the skin area and that the specific reactivity of the skin may be completely exhausted for 3 days or longer. In hay fever Mackenzie (39) has obtained temporary or complete relief by combining subcutaneous injections with the application of weak solutions of pollen extract, one containing only 0.001 mg. of nitrogen per hundred cubic centimeters, by nasal spray. Gradual increase of the strength of the solution in some instances produced tolerance to solutions one thousand times stronger than the one used at first. Caulfield (40) obtained similar results.

Hay Fever and Asthma. Hay fever is a special case of a respiratory idiosyncrasy in which the various pollens represent the responsible incitants. The conditions which are grouped together under the term of "hay fever" were associated with the inhalation of pollen by Blackley (41) as early as 1873.* Blackley also observed urticarial skin reaction when the material was rubbed upon the scarified epidermis, and catarrhal reactions upon instillation of pollen upon the mucous membrane of the nose and the conjunctivae. The supposition of Blackley concerning the relationship of pollen grains to the condition, was confirmed by Dunbar (42), who regarded the responsible constituent as primarily toxic. This view has now been eliminated, and it is clearly established that the disease is due to specific hypersensitiveness to some material in the pollen.

According to Scheppegrell (43), from 1 to 2 per cent of the population of the United States suffer from the disease at some period of the year. A considerable variety of plant pollens may be responsible. There was at first considerable difficulty in identifying the various inciting pollen weeds. This was due to the fact that many pollens are wind-borne, and often a common weed like the goldenrod, because of its profusion in the neighborhood of the patient, was held responsible, in spite of the difficulty with which its pollen grains are distributed, while the less conspicuous ragweed was neglected. Scheppegrell characterizes hay fever weeds as those which are wind-pollinated, very numerous, without bright color, or scent, and in which pollen is formed in great quantities. All such weeds, he says, are suspicious as possible causes of hay fever. The most important are the common ragweed, and the giant ragweed, in both of which the pollen is produced in such abundance that very slight winds will dislodge them and carry them in clouds to considerable distances. These weeds are responsible for probably 85 per cent of all cases of autumnal hay fever in the United States. Many grasses, however, can also give rise to hay fever. For a botanical survey of these, we refer the reader to Scheppegrell.

People who are susceptible to hay fever are usually attacked in the autumn, or the summer at a time when the particular weed to which they

* For historical literature see Cooke and Vander Veer (33).

are sensitive has reached the pollen stage. The disease appears gradually with signs of a cold in the head, itching and burning of the conjunctiva, and a catarrhal discharge from the nasal passages. In rare cases there may be fever and general malaise, fatigue, and depression. There are also cases of so-called "rose-cold" which occur in the summer, and others are characterized by onset in the spring.

Wolff-Eisner (44) was the first to suggest that anaphylaxis-like hypersensitiveness should be considered as a possible explanation of the condition.

The Antigen in Hay Fever. Much of the early controversy about the anaphylactic nature of hay fever turned upon the antigenic nature of the inciting substances in pollen. Failure to produce precipitating and complement-fixing antibodies in rabbits or anaphylactic conditions in guinea pigs by pollen injections induced Cooke, Flood, and Coca (13) and others to question the antigenic properties of pollen extracts. Parker (45, 46) in 1921 succeeded in sensitizing guinea pigs by repeated daily injections of pollen extract in large amounts. Alexander (47) sensitized guinea pigs with rye pollen. Coca and Grove (48) then showed that digested and dialyzed pollen still retained the property of arousing reactions in the skin, a result which suggests that this antigen may contain a partial antigen or haptene comparable to that present in bacteria. Walzer and Grove (49), however, succeeded in both sensitizing and producing anaphylactic reactions in guinea pigs with the trypsin digested and dialyzed pollen preparations. They believe that this non-digestible and non-dialyzable substance is probably the most potent antigenic constituent of ragweed pollen and suggest its being non-protein in nature. Ramsdell (50) later succeeded in sensitizing guinea pigs both actively and passively with pollen extracts. There can thus be no question about the fact that pollen contains a true and complete antigen capable both of sensitizing and of eliciting reactions.

In hay fever, as in other conditions discussed, local hypersensitiveness of the skin and of the conjunctivae often, though not invariably, runs parallel with respiratory sensitiveness, and has been used as an index for the diagnosis of the specifically responsible pollen.

Methods of preparing pollen solutions for diagnostic and therapeutic uses have been standardized and described by Grove and Coca (18).

Drug Hypersensitiveness. Drug tolerance, known for many centuries, develops after habitual use of alkaloids and inorganic poisons like arsenic, etc., and though the condition is similar to an immunity, evidence of an immunological mechanism is lacking.

So-called "drug idiosyncrasies" have been observed chiefly by dermatologists. Jadassohn (51) classifies them into those which appear on first contact with the drug and others in which repeated administration

is necessary to develop susceptibility. Acquired cases have been difficult to trace, yet sufficient evidence has accumulated to establish the possibility of the development of drug hypersensitiveness by habitual use. Bruck (52) has reported a case of acquired antipyrine susceptibility in a physician. Quinine hypersusceptibility has been reported by Dold (53) as developing in a definite though small percentage of workers in quinine factories. Similar reports have been made regarding workers in nickel factories. Untoward effects have frequently been noted in those receiving successive injections of arsphenamin (54, 55). Aspirin hypersensitiveness has developed in an individual within our experience. Ipecac sensitization in a pharmacist (56) and sensitization to azofuchsin in a factory worker have also been known to develop in the course of repeated exposure.

Pasteur Vallery-Radot (57) has reported a case of acquired hypersensitiveness to potassium chloroplatinate, which developed after three years of exposure.

In most drug idiosyncrasies the condition develops only after exposures of months and years, and in a small percentage only of exposed individuals — a fact which indicates the necessity for special conditions and perhaps special predisposition. This also may explain why it has been difficult to produce drug idiosyncrasies in animals.

There are reports in the literature of drug idiosyncrasy passively induced in animal experiments. Such reports largely emanating from clinicians (Bruck, Cruveilhier, Biberstein, and others) are rarely convincing, especially when they assert passive sensitization of animals with the sera of drug hypersensitive patients. Active sensitization of animals with simple chemical compounds has, however, been placed on a sound experimental basis and will be dealt with below.

The symptoms of drug hypersensitiveness are not mere intensifications of the normal physiological action of the drug. They most often take the form of skin eruptions but may, at times, exhibit respiratory and systemic symptoms closely analogous to those observed in other forms of hypersensitiveness. Cooke (58) reports cases of aspirin hypersensitiveness in which there were attacks of asthma following almost immediately upon ingestion of the drug.

Skin reactions have been observed in salvarsan hypersusceptibility, in certain cases of quinine idiosyncrasy such as those reported by Dawson and Garbade (59), and in many other forms of hypersensitiveness to simple chemically definable substances both in man and in animal experiments.

In drug idiosyncrasies the hypersensitiveness may be to the molecule as a whole, as is the case with antipyrine and some of the hypnotics or to certain radicals of the molecule. Doerr mentions cases of iodoform

hypersusceptibility in which the individuals reacted also to bromoform, thus placing the specific hypersusceptibility in the methyl radical. In the quinine cases of Dawson and Garbade the idiosyncrasy extended to levorotatory alkaloids such as ethylhydro-cupreine and cinchonidine, but not to dextrorotatory isomers such as quinidine and cinchonine.

It is plain that in those types of hypersensitiveness which are spoken of as drug idiosyncrasies the inciting agents are not antigens in any sense of the word. This was the difficulty which rendered it at first quite impossible to conceive of these phenomena as belonging in the same category or subject to the same mechanism as protein anaphylaxis. The situation has been clarified by the studies of conjugated antigens initiated by Obermeyer and Pick* and developed in so distinguished a manner by Landsteiner (60). The fundamental investigations on this subject have been discussed at length in the chapter on antigens. To recapitulate the principles briefly, it will suffice to recall the following facts:

It is possible to couple a great many simple substances with proteins in such a manner that the newly formed compounds are antigenic, their specificities oriented either entirely or in an overlapping manner by the simple substances which have been added to the protein molecules or by some of their radicals. The simple chemical substance or drug, in consequence, is in many ways analogous to the carbohydrate haptenes of certain bacteria and the lipoid substances of the Forssman antigen. In consequence, the conjugated antigen may be capable of producing antibodies and of sensitizing, and the simple substance or drug alone may, on the one hand, unite *in vitro* with the specific antibodies, producing the inhibition reaction of Landsteiner (60) which has been described in a previous chapter, or *in vivo* may, by itself, be capable of eliciting reactions. As applied to drug idiosyncrasy, this means that although the chemical substance unattached to protein might not in itself be capable of producing antibody formation, it might still be capable of reacting with the specific antibodies, once formed. The explanation of the drug idiosyncrasies, then, can be found in the fact that under special conditions encountered in predisposed individuals, repeated contact with the simple compound might lead to the formation of an antigenic combination of this compound with body protein; this antigen — because of the orientation of its specificity by the simple substance — is immunologically foreign to the body. It is thus capable of arousing some form of immunological response which, on subsequent contact, leads to reaction.

In a discussion of this group of phenomena, furthermore, it must not be forgotten that the older categorical identification of antigen and proteins is no longer tenable, apart from the haptene conceptions which have

* See Chapter II for references.

been discussed. Recent work has revealed that there are certain bacterial polysaccharides which by themselves may be capable of inducing a certain amount of immunity and determinable protective antibodies.

Experimental sensitization with simple substances has been observed for a long time. In 1911 Cash (61) experimentally sensitized human beings with an alkaloid obtained from satinwood, and Bloch and Steiner-Wourlisch (62, 63) in 1926 sensitized guinea pigs by skin application of the crystallized active principle of Primula ($C_{14}H_{18}O_3$). An instance of particular interest is that of the dye, ursol, commonly used for dyeing furs. The colored compound is an oxidation product of p-phenylenediamine, united with protein. Human sensitization, Landsteiner believes, can be assumed, in this case, to be brought about by a conjugated antigen comparable to azoprotein.

Experimental work based on the conception just outlined has been carried out by many observers, most convincingly by Landsteiner.

The importance of Landsteiner's * investigations in this connection consists in establishing on a sound experimental basis the fact that simple chemical compounds can be converted into antigens by being attached to proteins. The method found most useful for this purpose has been the preparation of azoproteins by coupling the proteins with diazonium compounds by the methods employed in the synthesis of azodyes. By diazotization of the aminobenzoic acids and the coupling of them to proteins, conjugates are obtained which, on injection into rabbits, cause the formation of antibodies. Such antibodies then react with all azoproteins made from the particular acid, irrespective of the origin of the protein employed for the coupling.

Landsteiner first found that, in dealing with the synthetic conjugate antigens, specific precipitation could be prevented by previous addition of the simple compound to the antiserum. For instance, the precipitation of the parabenzoic acid azoprotein by its corresponding antibody is inhibited by addition of a neutralized solution of the para-aminobenzoic acid. Obviously, this means that the acid combines with the antibody without leading to visible precipitation. Landsteiner was able to demonstrate the antigenic nature of the conjugate protein by actually shocking guinea pigs sensitized with an azoprotein by reinjection of the same substance or by other azoproteins prepared with the same diazonium compound.

Such experiments, however, do not entirely solve the problem of drug hypersensitiveness. It was still desirable to attempt the sensitization of animals directly with the simple chemical substances, unattached

* Landsteiner's studies have been discussed and references given in the chapter on Antigens. The work here quoted is based particularly on Landsteiner and Jacobs (64).

to protein, to determine whether in the body a combination might occur between these and the animal's own protein which would result in the formation of an antigen. That human beings are so sensitized by repeated contact with drugs has already been mentioned. This has been reported in connection with arsphenamine, phenylhydrazine, and dyes where the affinity for proteins of these substances might well account for the formation of antigenic products *in vivo*. Landsteiner approached the problem by the use of dinitrochlorobenzine, which was suggested to him by the frequent occurrence of allergic eruptions, reported by Wedroff (65), in industrial workers exposed to the compound. By injecting guinea pigs intracutaneously daily for eight days with 0.0015 mg. of this substance (a total of 0.02 mg.), Landsteiner elicited positive skin reactions, three weeks after the last injection, with a drop of a 1 per cent solution of the material in oil spread upon the skin. These experiments have been confirmed on human beings by Wedroff (66). On the basis of a study of seventeen chloro- and nitro-substitution products of benzine, Landsteiner makes the tentative suggestion that the power of these substances to sensitize is more or less proportionate to the velocity of their decomposition by alkali in alcoholic solution.

It is impossible to present all the experimental evidence available, but one may safely state that there are now many facts which indicate that so-called "drug idiosyncrasy" is really a drug hypersensitiveness in which the stimulating antigen consists of a compound in which the drug or the significant cleavage products of the drug form antigenic combinations with proteins of the host. The host thereafter becomes hypersensitive to the simpler drug alone, which represents a "haptene" not only to this chemical substance but to others as well, provided they contain the significant atom group.

Another interesting example of drug hypersensitiveness is that following upon the therapeutic use of the arsphenamines. It has been quite definitely determined that the occasional symptoms which follow repeated administration of the various salvarsan compounds are not due to or parallel with toxicity of the individual preparations. Those who have studied the problem most carefully agree that many of these manifestations represent a form of hypersensitiveness which depends not only upon individual idiosyncrasies of the patient but upon peculiarities of the particular lot of the drug employed. An excellent summary of the problem has been written by Stokes (67, 68). The manifestations most frequently observed are various skin eruptions, purpura, and late exfoliative dermatitis. There are other symptom complexes which are included as salvarsan hypersensitiveness by some observers but about which there is more or less disagreement, such as liver damage, hemorrhages in the brain, leucopenia, etc. Frei (54, 69), in 1928, was able to

produce skin hypersensitiveness to salvarsan in guinea pigs by a single intradermal injection of a small amount of the drug. There was difficulty in repeating Frei's experiments in America, but Sulzberger and Simon (70) were able to achieve sensitization of guinea pigs with relative ease and regularity by intradermal injection of neo-arsphenamine. The sensitiveness was apparently specific to the arsenobenzol complex and was not identical with hypersensitiveness to the element arsenic. There seemed to be some relationship between vitamin deficiency and the sensitiveness of the guinea pigs as determined by increased sensitiveness in animals that were not being fed on green feed. However, the environmental factors which are responsible for variability in susceptibility of the animals are uncertain. Again, it is interesting to note that intramuscular injections did not lead to sensitization in the same way in which application to the skin accomplished this; animals prepared by intradermal treatment did not show any signs of hypersensitiveness when subsequent administrations were made into the peritoneum or other parts of the body.

Observations of similar import have been made by Wormall (71) and, in our laboratory, by Jacobs (72, 73) and by Horsfall (74, 74 a). Wormall and Jacobs worked with iodized serum. The iodization is accomplished, according to Wormall's method, in dilute ammonium hydroxide solution, and the material purified by several precipitations with acetic acid and then redissolved by neutralization with sodium carbonate taken up in physiological salt solution, adjusted to *p*H 7.4. It appeared from these studies that rabbits immunized with such iodized serum produced precipitins which reacted with the iodized serum antigen, and such precipitation was inhibited by preceding addition of diiodotyrosine. Particularly important for the understanding of drug hypersensitiveness is the fact that, in Jacobs' experiments, three out of seven rabbits injected with iodo-rabbit serum responded with precipitin formation — in other words, the homologous protein of the animal became auto-antigenic on linkage with iodine. In later experiments by Jacobs the iodization was accomplished simply by allowing guinea pig serum to stand 15 minutes with two volumes of Lugol's solution at room temperature. Maximum precipitation was obtained with small amounts of glacial acetic acid and the precipitate taken up in weak sodium carbonate solution. With such Lugol-guinea pig protein it was possible to sensitize guinea pigs. Such sensitization was weaker than when the animals were treated with Lugol-horse serum, but nevertheless sufficient to indicate the possibility of sensitization with homologous iodo-protein.

Horsfall studied hypersensitiveness to formaldehyde. In the person studied exposure to minute amounts of uncombined formaldehyde by injection, immersion, or inhalation produced characteristic skin symp-

toms. Furthermore, injection of formolized proteins containing no uncombined formaldehyde produced positive skin reactions. Horsfall succeeded in producing antisera by the injection into rabbits of formolized rabbit serum. Antibodies produced in rabbits by formolized serum proteins were capable of passive sensitization as tested by the Dale method in guinea pigs. The uteri became sensitive to the homologous formolized serum, to the homologous native serum, and to heterologous formolized serum. Moreover, formolized serum proteins injected into rabbits produced a progressive and moderate skin hypersensitiveness to uncombined formalin.

Passive specific transfer of other forms of drug hypersensitiveness from man to animals or from man to man by the Prausnitz-Küster-reaction has not yet been successfully performed.

Given a rational and experimentally tenable mechanism to explain drug hypersensitiveness, we are still at a loss to account for the marked differences between individuals which determine the sensitization of a few where many are exposed. To some extent this may be explained by hereditary variation in the capacity to become sensitized — in the sense in which we discussed this question for hypersensitiveness in general. It may be, however, that other factors may be involved. Simon (75), in his studies on the poison-ivy sensitization of guinea pigs, found that a dietary deficiency in vitamin C inhibited the development of such hypersensitiveness. The problem of the relationship of vitamins to immunologic responses in general has had much attention but far too little precise investigation.

Hypersensitiveness to poison ivy, sumac (Dermatitis venenata), to primrose, and to certain other so-called "toxic" plants is in many respects similar to drug hypersensitiveness. The toxic principles of poison ivy and sumac have been shown to be antigenic, the latter demonstrated to be a glucoside (76). The active principles are extractable in 95 per cent alcohol and as Spain (77) has shown will produce an eruption if applied to the intact skin, after an incubation period of from 2 to 14 days. Spain showed that the typical lesion could be produced in 64 per cent of 104 cases and that the extract is non-toxic for animals. Heinbecker's (78) investigations on the Eskimos south of Baffin Land showed this race, entirely unexposed to contact with poison ivy, to be completely insusceptible, 65 consecutive tests being negative. These people, according to anthropologists and according to the blood grouping studies of Heinbecker and Pauli (79), are closely related to or identical with the American Indians. In the latter race, Deibert, Menger, and Wigglesworth (80) have shown that 56 per cent are poison ivy susceptible.

These observations form a reasonably clear argument that poison ivy susceptibility is not hereditary, but is a matter of exposure or contact,

a point in favor of bringing this type of reaction into harmony with other forms in which an immunological mechanism is responsible. To make the analogy still closer with the other idiosyncrasies, Schamberg (81, 82) has shown that desensitization to poison ivy is possible. In a discussion of eczema in Detroit, he stated that he had been able to protect persons susceptible to poison ivy by internal administration of minute, increasing doses of the tincture of Rhus. Later, Strickler made an alcoholic extract of the plant and by subcutaneous injections of aqueous dilutions of this extract prevented attacks in susceptible people. He reports cases which he treated in the following way: Tincture of Rhus was diluted, 1 cc. to 100 of sirup of orange and 5 cc. of rectified spirits, and beginning with one drop, 2, 3, 4, 5, etc., drops of the mixture were taken with each subsequent meal throughout a number of days, until 21 drops had been taken at a dose. Immunity was established after one month and lasted for about two months.

Active sensitization of animals with primrose extracts has been accomplished by Brunsting and Bailey (83); and Simon, Rackemann, and Dienes (84) have done the same thing with poison ivy. In both cases it was shown that extracts of the respective plants applied to the skins of guinea pigs resulted in specific hypersensitiveness which manifested itself by redness and swelling at the site of a second application. In subsequent experiments by Simon (85), it was shown that only applications to the skin sensitize successfully. Intravenous, intraperitoneal, or intramuscular injection gave negative or extremely feeble results. Passive sensitization was not possible.

A curious feature of such investigations is the fact that when sensitization is accomplished by skin application, the skin as a whole becomes sensitive. When the area of the skin to which the sensitizing application has been made is excised after 18 to 24 hours, such excision does not prevent the development of the general skin sensitiveness. Excision of the site within a few hours after application does, however, prevent generalization on the skin. Since injection into the blood stream and into the peritoneum did not sensitize, we must conclude that the epidermis, as an organ, plays an important role in the distribution of the skin sensitiveness. What the particular route or underlying principle of such distribution may be has not been determined.

It is apparent that there are gaps in the analogy of drug hypersensitiveness with the phenomena of protein anaphylaxis. Nevertheless the work of Landsteiner and studies such as those made with formaldehyde, iodine, and salvarsan render it certain that hypersensitiveness to drugs falls, in its main features, into the category of anaphylaxis and that there is no fundamental reason for segregating these phenomena into entirely different biological classes. We refer again to the paragraphs

at the beginning of our discussion of human hypersensitiveness, in which we called attention to the fact that differences of manifestation depend in all likelihood upon differences in chemical and physical structure of the responsible antigens. It seems well established both by chemical observation and by experimental analysis that all specific hypersensitiveness, including drug allergies, may be looked upon as belonging to the general class of biological manifestations in which the body is specifically changed by previous contact, the change expressing itself in an increased reaction capacity mediated by functional units which we speak of as antibodies and which may be determinable in the circulation or may exist fixed in the tissues and determinable only by the increased energy of reaction of the sensitive cells to contact with the antigen. This specific hypersensitiveness is a phase of the same mechanism which is active in immunity and which, although potentially harmful under circumstances which govern the manifestations of hypersensitiveness, is at the same time and essentially the mechanism which under other circumstances is responsible for the neutralization of toxins and for protection against infection.

The Special Function of the Skin. It is gradually being recognized that the skin, far more than a mere protective covering of the body, is a separate and important organ, with physiological functions of its own that affect the body as a whole in a variety of ways. The chemical structure of the skin has been studied in considerable detail, but relatively little is known about its functions other than those connected with its property as integument. That the skin has chemical functions of importance, however, is apparent from investigations such as those of Folin, Trimble, and Newman (86), who determined a rapid accumulation of sugar in the skin of animals when glucose was injected intravenously. The sugar concentration in the skin rapidly became almost equal to that in the blood. Trimble and Carey (87), later studying human skin from normal and diabetic subjects, found that elevation of sugar concentrations in the blood was accompanied by an absolute increase of the sugar in the skin, an increase far greater than that determinable in muscle tissue.

A number of observers have attempted to analyze the nitrogenous components of the skin, but the only investigations which we can find that seem to have bearing upon the present problem are those of Jena (88), who isolated, by a method which he does not describe in detail, a nitrogenous substance — possibly a protein or polypeptid — which contained tyrosin, tryptophane, phenylalanin, glutamic acid, and a very high percentage of cystin. The cystin content ranged between 7.2 and 13.2 per cent. Jena believed that his substance was similar in many respects to glutathion. He attributes to this skin derivative an extraor-

dinary reducing power and the property of protecting pigeons against a variety of poisons such as potassium cyanide, strychnine, phosphorus, histamine, and even diphtheria toxin. Although the reports of Jena are not entirely convincing and require further study, he has accumulated considerable evidence which suggests that by virtue of its chemical composition the skin may possess the function of removing toxic substances introduced into the body, and suffers injury as a result of this capacity of fixing the circulating poisons. This accounts, he believes, for a variety of dermal reactions such as toxic erythema, urticaria, etc. We mention this work not because we feel that it carries much conviction at present, but because it suggests lines of investigation which promise light on the position of the skin in connection with infection and immunity.

The thought that the skin possessed such immunological functions was first suggested by clinical observations concerning the characteristic involvement of the skin in acute general infections such as measles, scarlet fever, smallpox, etc. There were, furthermore, clinical opinions which have become traditional that syphilis which severely involved the skin was not likely to show later neurological lesions, and that skin tuberculosis was rarely associated with pulmonary lesions. The literature on this subject has been summarized by Tufts (89), who himself investigated the question of whether the skin was more active than other tissues in antibody production. Such investigations have not had uniform results. Many observers, including Tufts, found that intradermal injections of such antigens as typhoid vaccines did actually lead to quantitatively higher agglutinin contents in the blood than did the subcutaneous or intravenous inoculation.

We can hardly look upon any of these investigations as furnishing more than interesting suggestions for further study. There is, however, no doubt about the fact that the skin as an organ appears to occupy a more or less unique position in regard to hypersensitiveness. Although there have been rare instances of hypersensitiveness in which cutaneous reactivity has been absent, specific skin reactions accompany practically all forms of hypersensitiveness whatever the localization of the particular variety and whatever the antigen. The Prausnitz-Küster reaction, whether we regard it as a separate phenomenon or as a variant of ordinary antibody reactions, furnishes evidence that local sensitization with fixation of the antibody-like substances can take place in the skin. In most, if not all, drug idiosyncrasies the skin is either much more sensitive than other parts of the body or may be the sole site on which reactions can be elicited in the sensitive subject. Moreover, the cited work of Simon with poison ivy, or Sulzberger with arsphenamine, and Landsteiner's observations with dinitrochlorobenzene have shown that

repeated application to the skin eventually sensitizes the entire skin; and in some of these investigations there is reliable indication that methods of application other than the skin, such as intraperitoneal injection, either fail to sensitize either the skin or the animal as a whole or produce comparatively feeble effects. Simon's studies have shown that excision of the treated areas of the skin within 18 hours after application may prevent generalization of the skin sensitiveness. In all these facts, though too incomplete to form premises for theory, there is the strong implication that the skin possesses special functions that are still to be elucidated.

How generalized skin sensitiveness spreads from a single site is obscure. The lymphatics of the skin according to Hudack and McMaster (90) consist of a large plexus in the superficial layers of the corium which is horizontally continuous so that dyes injected in one spot may spread through abundant anastomoses to considerable distances. Moderate irritation or inflammation, slight stroking, or wheal formation increases the permeability so that the material introduced passes through the lymphatics as though no barrier existed. However, in addition to this horizontal distribution the dyes drain rapidly into the deeper lymphatics. It is a little difficult to conceive that the entire covering of the skin can be sensitized by horizontal diffusion of materials through the lymphatics of the corium. This is one of the matters that must be further investigated in explanation of some of the circumstances described. The possibility must also be considered that the skin by virtue of a characteristic chemical composition may possess a particular capacity for the fixation of many antigenic substances and a consequent increased tissue susceptibility to sensitization.

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CHAPTER XVI

HYPERSENSITIVENESS, *Continued*

Bacterial Hypersensitiveness

ANAPHYLACTIC reactions were obtained with bacterial extracts of colon, tubercle, anthrax, and typhoid bacilli by Rosenau and Anderson (1), and their experiments were confirmed by Kraus and Doerr (2), Holobut (3), Delanoë (4, 5, 6, 7), and others.

It became clear from the beginning that there were two distinct types of bacterial hypersensitiveness, one which corresponded to protein anaphylaxis, the other, typified by reactions like tuberculin and mallein hypersusceptibility, which seemed to differ from the anaphylactic phenomena. We must therefore discuss bacterial hypersensitiveness under two headings: (1) True Bacterial Anaphylaxis and (2) Bacterial Allergy, a term which we retain chiefly because "allergy" has been the word habitually applied to these reactions and because it is just as well to use a separate term in view of the fact that there is still considerable uncertainty regarding their precise mechanism.

Bacterial Anaphylaxis. The first observations on the so-called tuberculin reaction were made by Koch long before anything was known of anaphylactic phenomena. When the work of Otto and of Rosenau and Anderson revealed the fundamental principles of hypersensitiveness in general, the tuberculin reaction was interpreted as belonging to this group of phenomena. Even at this time the studies of Baldwin indicated that there were important differences between the tuberculin type of reaction and anaphylaxis, since he found that mere introduction of dissolved tubercle bacillus extracts did not render animals subject to typical tuberculin sensitiveness, but that the development of this reaction was intimately related to the formation of tubercles in actual infection. The differentiation of this reaction from anaphylaxis was established beyond question when Zinsser (8), in 1921, found that the two types of hypersensitiveness, that is, typical anaphylaxis to tubercular protein and the characteristic tuberculin allergy could exist in guinea pigs independent of each other or could be separately present in the same animal at different times. Thus, while guinea pigs suffering from tuberculosis would give a marked skin reaction to O.T. 1–10 within 10 days after inoculation, they did not develop uterine hypersensitiveness, in the anaphylactic

sense, until 5 and 6 weeks after infection and sometimes did not develop it at all. This led to an attempt to separate from the tubercle bacillus the individual antigenic fractions responsible for each reaction, an effort which resulted in the differentiation between nucleoprotein and a substance at first called the "residue antigen" which was analogous to the non-protein specific substance found by Pick (9) in typhoid bacilli and by Dochez and Avery (10) in the urine of pneumonia patients. Zinsser and J. T. Parker (11) then demonstrated similar "residue" substances in other bacteria — staphylococci, influenza bacilli, pneumococci, etc. These are the substances later purified by Avery and Heidelberger (12) and others and found by them to be carbohydrates which are type specific. These carbohydrate haptenes have been discussed in previous chapters. The subject, as far as tuberculin hypersensitiveness is concerned, was further clarified when Mueller (13, 14) and Laidlaw and Dudley (15) showed that the material that gave the typical tuberculin skin reaction could be separated by simple methods of precipitation from the antigenic materials which reacted *in vitro* with antitubercle bacillus sera.

There exists, then, a true anaphylactic sensitiveness to the materials of the tubercle bacillus which is in every way analogous to anaphylaxis in general and comparable to bacterial anaphylaxis with other organisms. Thus, tuberculous guinea pigs three weeks or longer after infection may develop typical anaphylactic reactions demonstrable by all the ordinary techniques, including the isolated uterus method of Dale, upon contact either with extracts of the tubercle bacillus or with tubercle bacillus carbohydrates. Enders (16) was able to sensitize guinea pigs passively with antitubercle bacillus rabbit serum and elicit typical anaphylaxis with tubercle bacillus carbohydrate.

It is clear, then, that true anaphylaxis can be produced with tubercle bacillus materials as with the antigenic substances of other bacteria, but that animals so sensitized may not show any sign of that form of hypersensitiveness which is spoken of as allergy.

Bacterial Allergy. The form of hypersensitiveness to which we apply as a matter of usage the phrase "bacterial allergy" is not limited to the tuberculin reaction. It occurs in glanders (mallein) and in abortus infections, in both of which it is of some diagnostic value. It has been experimentally produced by Zinsser and Grinnell with pneumococcus and is, in our opinion, a general reaction of some pathological importance in practically all bacterial infections. The question of its general significance will be discussed below. For the present we will limit ourselves to the discussion of the tuberculin reaction since it is the most commonly observed and typical of these phenomena.

The basic observation leading to the recognition of bacterial allergy

was made by Koch (17, 18) upon guinea pigs. He describes his observation as follows:

"Tuberculin may be injected into normal guinea pigs in considerable quantities without causing noticeable symptoms. Tuberculous guinea pigs, on the other hand, react to comparatively small doses in a very characteristic manner."

Since, in Koch's experiments upon tuberculin, it was desirable to obtain sharp reactions, he did not content himself with the production of moderate symptoms by the injection of slight amounts of tuberculin into infected animals, but increased his dosage until the guinea pigs were killed. He showed that guinea pigs having a moderate infection — 4 to 5 weeks after inoculation — could be killed by doses of 0.2 to 0.3 gram, while animals in advanced stages would succumb within 6 to 30 hours to quantities as small as 0.1 gram subcutaneously. In the animals so studied he determined not only a systemic effect, but a local reaction in the skin, areolar tissues, and adjacent lymph nodes.

Koch's observations upon guinea pigs were applied by him, Guttstadt (19), Beck (20), and others to man, and the result was the development of the important diagnostic test. The fundamental fact in this as well as in other tests of this kind, is the local and systemic reaction of infected subjects to contact with antigenic material which, in the same quantities, produces no effects in normal individuals. In this sense, then, the phenomenon is one of specific hypersensitiveness.

Koch's original interpretation of the phenomenon was that tuberculin contained substances which caused tissue necrosis. The necrotizing action was particularly powerful upon tissues already saturated with the toxic material.

Similar to this view is the one later expressed by Babes and Proca (21), who attribute the systemic symptoms to a sudden lighting up of the existing lesions by the tuberculin.

The first suggestion of an immunological mechanism was made by Wassermann and Bruck (22). They accepted Ehrlich's assumption that certain cells of the tuberculous foci (those situated just below the periphery and already affected by the tubercle toxin, though still resistant) were possessed of an increased receptor apparatus for the tubercle antigen. For this reason the injected tuberculin was concentrated in these foci, attracted out of the circulation by the increased avidity of these cells, the consequence being increased activity of the lesions and systemic symptoms. The tuberculin reaction, according to these writers, therefore, would be caused by the union of the tuberculin with the "sessile receptors" upon the diseased tissues.

Important advances in the general understanding of reactions of this type came with the development of skin reactions by von Pirquet (23, 24, 25) and the ophthalmo reactions, by Calmette (26) and by Wolff-Eisner (27).

The first extensive studies of the skin reaction are those carried out by von Pirquet (23, 24, 25), by the method of placing drops of undiluted tuberculin (O.T.) on the forearm with a similar drop of 50 per cent glycerin as control and lightly scratching the skin through the drops. A positive reaction consisted of red, slightly raised nodules which appeared within about 24 hours after application, increased and changed in color, sometimes leaving a slight central hemorrhagic spot during the next 3 or 4 days. A large number of tests carried out at that time in Vienna on clinically normal children of various ages showed that positive reactions began to appear from the age of 2 with about 7 per cent positive and an equal number doubtful ones, up to 35 per cent positive and slightly over 40 per cent doubtful at the age of 14. Other investigators, carrying out similar tests in other parts of the world, obtained results of analogous significance. It must be remembered, however, that only a limited percentage of the positive children developed active tuberculosis and that the test is so delicate that it undoubtedly indicates latent and very mild infection. Investigations carried out by still more delicately quantitative methods since von Pirquet's first work was done have shown not only that the positive reaction is not necessarily of prognostic importance, but also that in the course of better hygiene and tuberculosis control, the range of positive reactions has been shifting to more advanced ages, so that children do not seem to be infected as early as they were formerly, and that the percentage of positives in children generally is decreasing.

The ophthalmo reaction which consisted in the installation of a 1 per cent solution of an alcohol precipitate of O.T. into the conjunctiva has given comparable results but is not often used today since some observers have suspected that the local reaction may serve to light up latent ophthalmic foci of tuberculosis.

One of the difficulties in earlier statistical studies of tuberculin reactions was the great fluctuation in potency of different specimens of Old Tuberculin. The separation of the allergic and anaphylactic antigens from tubercle bacilli and the consequent isolation of the active allergic principle of O.T. by Mueller and others have made it possible to achieve a certain degree of standardization. At the present time, most clinical investigators who carry out comparative tuberculin tests use some form of purified tuberculoprotein. Most of these preparations are made according to the method of Seibert (28, 29, 30), who grows the tubercle bacilli on an asparagin synthetic media containing 10 per cent glycerin, concentrates after heating, by filtration through a 13 per cent guncotton filter, and precipitates with trichloracetic acid. Details of the method may be found in the original articles. Of this purified tuberculoprotein, Long and Seibert (31) advise, for the first test dose, 0.00002 mg. in a volume of 0.1 cc. If negative reactions are obtained with this dose, a second test can be made with 0.005 mg.

Employing the more exact methods, Harrington and collaborators (32) carried out a large series of tests on children in Minneapolis in 1936 and compared these with similar tests done ten years before. The following table, which is constructed from the chart given in their publication, indicates a marked diminution of actual percentages of positive tests in the course of these ten years, as well as a marked shifting of the age group upward.

Age Group	*Positive 1926*	*Per Cent 1936*
6	20	13
6–7	28	15
7–8	40	12+
8–9	41	16
9–10	50	16
10–11	54	20
11–12	60	21
12–13	58–59	20
13–14	70	26

In estimating the contrast of these figures with the earlier ones of von Pirquet, it must be borne in mind that von Pirquet himself, commenting on his own tests, stated that his children all came from the poorer sections of Vienna where the tuberculosis rate was notoriously high.

With the method described above, Long and Seibert (31) compiled the reports of 18,744 tests carried out on university students in different parts of the United States in the general age group of 18 to 19 years. Their positive tests indicated a geographic difference in tuberculin positives, in that the rates were highest in the East, where, for instance in Pennsylvania, the positives in this age group were 42.9 per cent in women and 48.8 per cent in men; lowest in the Middle West, with 24.6 per cent in women and 24.8 per cent in men; and relatively higher on the Pacific coast, with 29 per cent in women and 36 per cent in men.

The studies of the antigenic properties of the tuberculin protein by Seibert (33, 34, 35) have, among other things, been suggestive of interesting relationships between molecular size and antigenicity (36). Seibert showed that the capacity to elicit tubercular skin reaction may reside in a whole series of molecules ranging in molecular size from about 2000 to 25,000. The smallest molecule was approximately equal in skin potency per milligram of nitrogen to the largest molecule. But the larger molecules were far more effective in the ability to elicit antibodies. The smallest ones were practically non-antigenic in this sense. Furthermore, Seibert showed that a purified tubercular protein (obtained by the precipitation of Old Tuberculin with trichloracetic acid) of low molecular weight, and skin reactive but not able to stimulate antibodies, became truly antigenic when injected in a particulate form after adsorption to aluminium hydroxide or charcoal.

Mechanism of Bacterial Allergy. Earlier theories of the mechanism of such reactions may be dismissed without much comment, since most of them, like those of Wolff-Eisner (37), of Calmette (38), and of Friedberger (39, 40), were based upon assumptions of the formation of a toxin by specific lysis.

Experimental study was begun by Römer (41) in 1909 and by Baldwin (42) in 1910. Baldwin's work is fundamental, in showing that guinea pigs could not be rendered skin-sensitive by implantation of porous filter capsules or celloidin capsules containing tuberculoprotein, or living tubercle bacilli. He asserted that skin sensitiveness could never be

produced without actual infection with living organisms. His conclusions can be summarized as follows: Tuberculous animals become sensitive to anaphylactic test, but not uniformly so. There is no absolute relation between the degree of sensitiveness and the stage of the disease. Injections of the tuberculoprotein may sensitize normal guinea pigs. Guinea pigs so sensitized, however, do not react to the ordinary tuberculin test.

Fleischner, Meyer, and Shaw (43) subsequently studied cutaneous hypersensitiveness in guinea pigs treated with intraperitoneal injections of *Bacillus abortus bovis*, *Bacillus typhosus*, and O.T., carrying out parallel experiments between animals treated with the living organisms and those with the dead, and confirmed Baldwin's observation that animals treated with bacterial protein extracts might become anaphylactic, but did not give the typical tuberculin-like skin reaction, thus generalizing the fact that inflammatory tissue reactions are necessary.

Passive transfer of tuberculin sensitivity has not so far been possible. There are a number of incomplete experiments in the literature which are suggestive, but not conclusive.

Such are the experiments of Bail (44), who claims that the injection of tissue mash of tuberculous organs created, 24 hours later, hypersusceptibility, with death after intraperitoneal injections of tuberculin. Helmholz (45) reported positive skin reactions in guinea pigs 2 to 6 days after he had injected them with defibrinated blood of tuberculous guinea pigs. Mueller and the writer (46) produced a moderate degree of tuberculin sensitiveness in guinea pigs by injecting with the serum of rabbits in which multiple tuberculous lesions had been produced. None of these passive transfers can be regarded as possessing anything like a convincing character or regularity of passive transfer.

In our own work (8) in which we were able to separate anaphylactic sensitiveness to tuberculoprotein and tuberculin allergy, in attempting to sensitize guinea pigs with the nucleoprotein fraction, we found at times, when the nucleoprotein was unfiltered, that tuberculin-like reactivity developed. As a result of this, Zinsser and Petroff (47) were able to demonstrate that dead tubercle bacilli could produce tuberculin reactivity as well as the living, provided a tubercle was formed. The filtered material did not produce allergy. This fact is important in demonstrating that the tuberculin reaction is not, as formerly believed, an infection reaction, but depends, as Baldwin had correctly surmised, upon the formation of inflammatory tissue reactions, the tubercles.

The only experimental exception to the rule "no tuberculin reaction without a tubercle" is the observation of McJunkin (48), who injected tubercle bacilli into the peritoneal cavities of tuberculous guinea pigs. When the animals had become very ill, in 24 hours, he filtered the peritoneal exudate and injected it into normal guinea pigs. After 6 or 8 days

the animals which had received the exudate reacted to tuberculin. This must be interpreted as an active sensitization without tubercle formation and without the use of whole tubercle bacilli. The experiment is not easy to repeat, has often failed in our hands, but cannot be neglected in a discussion of the reaction.

An observation of fundamental significance is that of Dienes (49, 50), who found, in 1927, that reactions indistinguishable from typical tuberculin allergy could be produced with ordinary antigens like egg white and horse serum if these substances were repeatedly injected into tuberculous guinea pigs. The experiment was most regularly successful if the first injection of the protein antigen was made directly into a tuberculous focus, either a gland or the testes. Dienes's (51, 52) experiment is not always successful, but it has been repeated with sufficient frequency to indicate that the observation is correct. There has been considerable experimentation in efforts to reveal the significance of this phenomena. Dienes (53) believes that the tuberculin type of hypersensitiveness represents the first stage of every immune response to parenterally introduced protein. He bases this to some extent upon histological studies carried out with Mallory. On the other hand there are alternative explanations and Dienes's experiment is not out of harmony with the view that the peculiar antigenic material which gives rise to the tuberculin type of sensitiveness is produced in the body from the tubercle bacillus by the products of the tissue reactions incited by bacteria in localized foci.

Hanks (54), who has studied the Dienes phenomena, confirmed the observations of Dienes and Schoenheit but found in addition that it was not necessary to inject the ordinary protein antigen directly into a well-developed tuberculous focus. His procedure consisted in injecting tubercle bacilli into one testicle of the guinea pig, removing this testicle on the following day and injecting 0.1 cc. of horse serum into the opposite testicle. Tested 10 days later with 0.01 cc. of horse serum intracutaneously, he obtained delayed allergic skin reactions of the tuberculin type. From this Hanks concludes that the tuberculin type of hypersensitiveness may be induced by ordinary protein antigens without any antigen-modifying influence on the part of the inflammatory tubercular reaction. It must not be forgotten, however, that in Hank's experiment, even though the infected testicle was removed 24 hours later, there must have been a considerable distribution of tubercle bacilli in the body with the consequent formation of small foci with which the subsequent injected horse serum inevitably came into contact.

In attempting to elucidate the principle, Zinsser and Grinnell (55) turned to the pneumococcus, an organism which autolyzes readily and from which it could be hoped that an allergic antigen, undamaged by chemical treatment, might be obtained by a biological process of dis-

solution, perhaps not unlike that brought about by the activities of inflammatory tissue cells on more resistant bacteria. It was found in these experiments that guinea pigs treated with large amounts of pneumococci or repeatedly injected with autolyzed pneumococci would in many cases develop skin sensitiveness to pneumococcus autolysates which appear late and are, if anything, more hemorrhagic and necrotic than severe tuberculin reactions. These results were analogous in every way to the tuberculin phenomenon except in the fact that it was not possible to kill animals so sensitized by intraperitoneal injection of the autolysates.

It is impossible, on the basis of experimental knowledge, to define precisely the mechanism of the tuberculin type of hypersensitiveness. We can approach an understanding, however, by a correlation of the following observed facts:

(1) Allergic skin reactions are sufficiently specific to indicate that the sensitizing antigen is derived from the substance of the tubercle bacilli.

(2) The sensitizing effect of dead bacilli excludes the participation of toxins or other unstable bacterial antigens produced in living cultures and not derived from dead cells.

(3) Failure of allergic sensitization with soluble extracts, O.T., or purified tubercular proteins in the absence of tuberculous foci indicates the importance of inflammatory cellular reactions in the mechanism of the sensitization.

(4) The Dienes experiment shows that in the body of tuberculous animals there is established a mechanism which can convert an ordinary anaphylactic sensitization into an allergic one; and the fact that the Dienes experiment succeeds best when the sensitizing horse serum or egg white injection is made directly into the tuberculous focus suggests the possibility that the mechanism is primarily one of antigen modification. The experiments of Hanks are somewhat against this but do not exclude this possibility.

To summarize, we may say that the facts, as far as they go, indicate that allergic sensitization, in the tuberculin sense, is a form of hypersensitiveness modified for all antigens by the presence of inflammatory cell reactions incident to bacterial infection. Although the phenomenon is most marked in tuberculosis, it extends to other forms of bacterial invasion.

The unsolved question regarding the mechanism turns upon the following points: Is such allergic modification dependent upon a change in the antigen injected into tuberculous animals and produced under the influence of the inflammatory tissue reactions; or is it, on the other hand, due to a general modification of the reaction capacity of the animal, so that the nature of the lesions, produced by the second antigen adminis-

tration, is dependent upon the same basic mechanism of sessile antibodies as in ordinary anaphylaxis but that now the antigen reacting with these sensitized, but physiologically altered cells produces the allergic type of response?

Bacterial Skin Reactions in Rabbits. Skin reactions in rabbits apparently follow laws that are somewhat different from those observed in guinea pigs, these differences perhaps furnishing a clew for a better understanding of certain forms of hypersensitiveness in general. Gay and Claypole (56), working with typhoidin, a preparation made from typhoid bacilli analogous to tuberculin, believe that the positive skin reactions obtained in rabbits were parallel with the degree of immunity of the animal, and have succeeded in transferring susceptibility passively by inoculation of 24 cc. of typhoid immune serum 24 hours before test. Meyer and Christiansen (57, 58) obtained reactions which are in substance similar to those of Gay and Claypole, though Meyer is not inclined to regard the hypersensitiveness as parallel with immunity, a view that corresponds with the later views on the significance of the tuberculin reaction. Julianelle (59, 60, 61), studying pneumococcus skin reactions in rabbits, finds that he can obtain reactions with pneumococcus nucleoprotein in rabbits sensitized with injections of heat-killed pneumococci. Julianelle also found that passsive sensitization was possible. An interesting phase of Julianelle's experiments is the fact that the intracutaneous injection of heat-killed pneumococci confers not only skin hypersensitiveness, but also conjunctival hypersensitiveness. These eye reactions were not passively transferable.

Bacterial Allergy and Infectious Disease. When, in 1923, we studied, with Parker (11), the different phases of bacterial hypersusceptibility, we suggested that, in the course of any infection in which bacterial foci are formed, there follows the development of "a type of hypersusceptibility which is distinct from protein anaphylaxis" which can be determined by intradermal skin reaction, and which might explain the remote injuries in infections in which bacteria that were not true exotoxin producers were concerned.

This point of view was strengthened in subsequent studies on streptococcus and pneumococcus allergies. As a consequence the allergic mechanism has been considered as possibly concerned in the pathology of a number of important diseases of man.

The following paragraphs set forth the relationship of the allergic phenomena to the pathogenesis and immunity of a few of the more important diseases in which this question has been considered.

Allergy and Tuberculosis Immunity (64). The significance of bacterial allergy in tuberculosis has been the objective of a long series of investigations begun, in principle, by Koch in his studies on super-infection. The subject has been reviewed, from a slightly different point of view, by Long (62). Though Long deals particularly with the development of methods of immunization, he discusses at some length the view that the

allergic state is "nothing more than a rapid mobilization of the same forces which operate in the normal animal." Löwenstein (63) quotes von Behring, Escherich, and others as having favored this view.

There has been a great deal of discussion concerning the relationship of tuberculin allergy with immunity to tuberculosis. In this particular subject investigation has moved around in circles for many years with the continuous rediscovery of old facts and no agreement whatever. It is impossible to review the literature, since an enormous number of papers have come out on this subject. Experiments done by Zinsser, Ward, and Jennings (65) some years ago supported the earlier views of Kraus, Löwenstein, and others that guinea pigs rendered allergic (in our own experiments with dead tubercle bacilli) resisted the progress of tuberculous infection and remained alive longer than normal controls. It was and still is our opinion that the allergic reaction is an integral part of the specific immune response and represents an increased reaction capacity of the protective mechanism to contact with the invading organisms. Rich (66, 67) has of late years been the chief proponent of the opposed view, mostly on the basis that allergy does not necessarily parallel immunity.

These matters are more completely discussed in the chapter on tuberculosis immunity. However, the subject is important in connection with the question concerning the possibility of desensitizing against tuberculin allergy, a principle which is important for infections in general as well as for tuberculosis. In other words, can an "allergy" go on to an "anergy" in which a hypersensitiveness of the immune response has been converted into a condition of complete local resistance? Many efforts have been made to desensitize tuberculin reactive guinea pigs, in most cases without success. A thorough study was made in 1934 by Rothschild, Friedenwald, and Bernstein (68) in which they came to the conclusion that complete desensitization to tubercle bacilli and to tuberculin can be achieved by long-continued daily subcutaneous injections of doses of old tuberculin. These experimental observations are not unlike in principle to those made by Swift and his co-workers with streptococci.

Allergy and Streptococcus Infections. In 1925 Dochez and Sherman (69) and Zinsser and Grinnell (70) published studies in which sensitization with streptococcus cultures and with filtrates of the so-called "Dick toxin" was carried out. Sensitization was most successful when the whole bacteria were injected, but other streptococcus products — including the Dick filtrate — likewise induced sensitization. The most potent reactions on reinjection were obtained with the Dick filtrates, which are analogous to tuberculin, in that they represent a filtrate of cultures in which a certain amount of autolysis may be regarded as having taken place and which is both heat stable and allergically active. This

assumption is supported by the work of Duval and Hibbard (71), who found that the culture lysate produced from scarlatinal streptococci *in vivo* was a more potent antigen than the ordinary culture filtrate.

The chief differences between our own studies and those of Dochez and Sherman (69) consisted in the fact that they were able to neutralize the allergic antigen by the use of antiscarlatinal serum. We did not succeed in this, as we had failed previously in many attempts so to neutralize tuberculin with antitubercle bacillus sera. However, we used guinea pigs, whereas Dochez and Sherman worked with rabbits. A later publication by Dochez and Stevens (72) confirms the earlier studies regarding both the production of the allergic state and neutralization with serum. They found, moreover, that rabbits pass through several consecutive periods: One of sensitiveness, during which the animals display a cutaneous sensitiveness which can be neutralized with serum; following this a second period of hypersusceptibility supervenes, during which no neutralization is possible; finally the animals become entirely insensitive.

Dochez and Stevens interpret their results as lending likelihood to the suggestion made by Bristol (73) that the rash and the clinical symptoms of scarlet fever may be due to allergic sensitization to the products of hemolytic streptococcus. They call attention to the fact that in early infancy intracutaneous injections of the scarlatinal filtrates are usually negative, and Cooke (74) adds to this the important observation that the blood serum of such negatively reacting infants has no neutralizing power. It is therefore at least important to consider the possibility that sensitization to an organism which so frequently invades the human throat may take place and thereby give rise to an allergic condition to which many manifestations of disease could be attributed. Investigations of similar significance have been carried out by Mackie (75) and by Smith (76).

Ando, Kurauchi, and Nishimura (77) believe that the so-called Dick filtrate contains both an allergic and a primary toxic constituent.

Allergy and Rheumatism. Clinical evidence has established the association of tonsillitis, injuries of the myocardium, and chorea with the various pathological conditions known as acute and subacute arthritis. The clinical course of acute rheumatic conditions is such that it suggests an infectious etiology, and in the associated conditions of tonsillitis and cardiac lesions, bacteria of the streptococcus group have frequently been found. Yet, though bacteriologists have taken frequent cultures from joint exudates and from pericardial fluid, positive results have been the exceptions. The opinion that there is no actual bacterial invasion of the joints themselves is borne out by the clinical course and by the pathological histology of rheumatic lesions.

It is fair to formulate from this that, while acute articular rheumatism and associated conditions, in their ultimate etiology, are probably bacterial infections, the actual condition of the joints themselves is not the result of local invasion.

As to the underlying bacterial causation, the majority of investigators — guided particularly by the result of bacteriological studies of throat lesions, foci in the teeth, blood cultures, and cultures from valvular vegetations — have continued to attribute rheumatism to some form of streptococcus. The most extensive work on this problem is that of Swift and his associates. Swift and Kinsella (78) in 1917 found non-hemolytic streptococci in less than 10 per cent of the blood cultures of rheumatic cases. They proved that the organisms recovered from the patients did not fall into a uniform group and that there is no justification for speaking of a "Streptococcus rheumaticus." Swift and Boots (79) then succeeded in producing a large number of joint lesions in rabbits with four different strains of non-hemolytic streptococci and subsequently Swift and his co-workers, as well as Zinsser and Yu (80) found that not only were organisms isolated from rheumatic cases serologically heterologous, but that there might be two different types of streptococci in one and the same case. In the cases studied the organisms were isolated after death from heart muscle and spleen, though blood cultures in life had been sterile.

The idea of allergy as an explanation for the rheumatic symptom complex was introduced by Herry (81) and experimentally investigated by Faber (82) as early as 1915. Faber's work suggested experiments carried out by Zinsser and Wu (83), in which joint lesions could be produced with horse serum, but only by direct repeated injection of the joints themselves. Zinsser and Grinnell (70) then produced severe allergic reactions with streptococci and pneumococci in guinea pigs, finding parallelism between skin hypersusceptibility and joint susceptibility. They never succeeded in producing joint lesions by anything but direct joint inoculation.

At the same time studies of allergy in rabbits with green streptococci were being carried out by Swift and Andrewes, Derrick and Hitchcock. Swift and his collaborators (84) have not only made interesting additions to the knowledge of streptococcus allergy in rabbits, but have established proof of the hypersensitiveness of rheumatic patients to streptococcus nucleoproteins. Andrewes, Derrick, and Swift (85, 86) made the observation that when green streptococci were intradermally injected in susceptible rabbits, a primary lesion occurred which retrogressed after about 48 hours and was followed by a secondary reaction in about 8 or 9 days. McDonald (87) has favored the allergic conception on the basis of similar observations.

The allergic view of rheumatism may be taken to rest at the present time upon the following premises:

1. In acute and subacute rheumatic conditions the joint fluids are usually sterile, though signs of inflammation are present.
2. The organisms isolated from rheumatic cases are not bacteriologically or serologically identical.
3. Streptococcus foci are found in many rheumatic cases.
4. Joint lesions can be produced in animals with products of these organisms, and such joint hypersusceptibility can be shown to some extent to be parallel with skin allergy.

Swift (88) summarizes the subject as follows:

". . . the so-called allergic theory does not establish unequivocally the etiological relation of streptococci in rheumatic fever, but only furnishes us with the best explanation of how different strains can all induce a similar clinical and microscopic picture."

Toxin Hypersusceptibility. Long before there was any knowledge of anaphylaxis, von Behring (89) observed that animals that were being actively immunized with tetanus and diphtheria toxins developed a degree of susceptibility to these poisons greater than normal. Behring spoke of "Gift Ueberempfindlichkeit" as a property acquired as a result of a preceding injection. The observation was confirmed by Knorr (90) in 1895 and by von Behring with Kitashima (91), a few years later. They showed that guinea pigs repeatedly treated with small doses of toxin may sometimes fail to show immunity and develop a susceptibility so high that doses too small to injure normal animals may cause death. Similar observations were made upon horses by Salomonsen and Madsen (92) and by Kretz (93). The so-called "paradox phenomenon" of Kretz was much discussed and Wassermann (94) attempted to explain it by assuming an increased receptor apparatus, the receptors remaining attached to the cells, thus rendering the cell more susceptible to toxin than before. Objections to this theory arise from the observation of similar hypersusceptibilities in animals whose blood contains free antitoxin. Moreover, Friedemann (95) quite justly objected that toxin hypersusceptibility as observed by the writers mentioned represented an intensified toxin effect and not one of hypersensitiveness in the accepted sense. Until quite recently, therefore, it was questioned whether such a thing as true toxin hypersensitiveness existed.

In 1927 Neill, Fleming, and Gaspari (96) advanced evidence to show that the primarily toxic antigens possessed the same fundamental immunological properties as other antigens. Later Neill and Fleming (97) succeeded in producing symptomatically typical anaphylactic reactions in guinea pigs actively sensitized with diphtheria toxin and anatoxin, as well as in animals passively sensitized with antitoxic sera derived

either from guinea pigs or from man. They were quite aware of the fact that the formalinized diphtheria filtrate, even after acid precipitation, still contained other derivatives of the diphtheria bacillus besides the toxic principle and that, similarly, antitoxic serum may have and often did contain antibodies against diphtheria protein. Nevertheless, their evidence as a whole indicated that the toxin or toxoid antigen was the principal factor in the active sensitization of the guinea pigs. In passive sensitization experiments it was possible to remove all detectable traces of other antibodies without removal of the antitoxin, by absorbing the antitoxic sera with heavy suspensions of the bacilli. Such absorption removed from the sera all precipitins and all capacity for sensitization against nucleoprotein solutions. Their experiments, therefore, appear to be clear in demonstrating that the sensitizing capacity of the absorbed antitoxic serum and the subsequent shock produced with toxin or toxoid solutions definitely represented an anaphylactic reaction in which the reacting factors were toxin and antitoxin.

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CHAPTER XVII

IMMUNITY IN ULTRA-MICROSCOPIC VIRUS DISEASES;* RICKETTSIA DISEASES; PROTOZOAN INFECTIONS

Principles of Immunity in Virus Diseases. There are still so many uncertainties in our knowledge of the virus agents that it is necessary to be extremely cautious of generalization. However, the fact that these agents are antigenic in the sense that they can incite specific reaction bodies analogous to antibodies has been ascertained with certainty for all of those that have been immunologically studied.

Before considering the immunology of virus diseases it will be useful to discuss a few of the pertinent facts that are known about the virus agents themselves.

In size they vary from the very small dimensions (10 to 100 mμ) in poliomyelitis, louping ill, and plant mosaics, to the relatively large magnitudes of 200 mμ and over. If we accept the so-called elementary bodies of vaccinia and varicella, the Paschen bodies, the Borrell bodies of fowl pox, and some of the others observed by Ledingham (1) and his associates in varicella and in zoster as representing the virulent agents themselves, we are confronted with magnitudes just within the range of visibility after staining.

The manifold nature of the diseases of plants and animals caused by these agents, their differences in manner of infection, symptomatology, and epidemiology, indicate that we have to do with phenomena as diversified as those of the bacterial diseases. Like the bacteria, they have certain fundamental similarities, but they differ from one another to such an extent in specific details that observations made with one should not be carelessly generalized to the group as a whole. The crystallization of the mosaic virus by Stanley (2, 3) appears to indicate that virus agents are inanimate protein complexes. Until Stanley's discovery, no very significant progress was made concerning the living or inanimate nature of the virus agents. This work seems to have thrown the scales heavily to the side of the opinion that they are inanimate protein complexes, in this case, of molecular weights ranging from 5,000,000 upward,

* Much of this discussion is taken from Zinsser (5), "Immunologic Consideration of the Virus Problem."

in contrast to molecular weights of about 30,000 to 60,000 for the protein of the uninfected plants. It is still possible, of course, to object that the process of crystallization is not necessarily conclusive, that it is difficult, for instance, to purify amino acids by repeated crystallization and that the true virus of mosaic might still be an impurity on the crystals themselves around which crystallization has taken place. Yet Stanley's quantitative measurements seem to show that the yield of virus is proportionate to the concentration of total protein of the plant juice in the crystals. For the immunologist, supposing that virus agents are inanimate large protein molecules, it is necessary to assume that the multiplication of the virus substances in the living cells of the host tissues resulted from a chemical transformation or synthesis of the host's protein, and that the new substance becomes antigenically heterologous, that is, auto-antigenic. Such auto-antigenic power is by no means unheard of, since it has been invoked, on a reasonable experimental basis, in analogy with heterophile antigens, in explanation of the Wassermann reactions, and has been reliably determined in connection with drug hypersensitiveness. In order to accept this view, it is necessary to assume that the same stimulus can induce the formation of chemically and physically identical proteins in a number of different tissues and in different animals. Thus, for instance, yellow fever virus which multiplies in the viscera of man would have to be identically reproduced by the nervous tissues of mice and by the tissues of mosquitoes. And the neurotropic yellow fever virus in mice, though not reversible to the original condition, yet immunizes monkeys and man in such a manner that we are forced to assume its chemical identity with the original substance produced by the cells of man, monkeys, and mosquitoes, in spite of otherwise profound biologic alteration. Similar difficulties arise in regard to vaccinia protection against variola and with *virus fixé* protection against street virus of rabies, etc. These are difficult biologic hurdles to take, but in the light of Stanley's work, they may have to be taken. In this connection it is interesting, and to an extent favors Stanley's contention, that the multiplication of virus in tissue culture proceeds during the period of active tissue metabolism. When metabolism of the supporting cells comes to a standstill, the virus not only ceases to grow but deteriorates rapidly (4).

It can, of course, be argued that filterable virus agents are not all necessarily alike. That, of course, may be granted, but while these agents may differ in a great many secondary chemical and biologic attributes, there are still so many fundamental similarities, especially immunologic ones, that it is very difficult to conceive one group as inanimate complexes, and the other as living entities.

In approaching the immunologic problems, it is well to consider those

generalizations which it appears safe to postulate about the virus agents and which may have bearing on the immunologic questions. Those which seem most important to us are the following: With no reliably demonstrable exceptions the virus agents multiply only within or in close association with the living cells which they damage. None of the virus agents have so far been cultivated outside the animal bodies except in the presence of living or viable susceptible cells. It is our own belief, based on experiment, that they can multiply only with the co-operation of the invaded cell. The entire theater of reactions, therefore, is transferred to the interior of cells of limited distribution, since the selective specificity of the virus agents is usually a much higher one than is the case with bacteria. Moreover, the virus agents appear to be biologically more flexible than are bacteria. It is well known that a large number of agents, which in spontaneous infection have no selectivity for the nervous system, can be rendered neurotropic, and once so adapted they may, as in yellow fever, herpes, and Nicolas-Favre disease, be extremely resistant to reversion to their original conditions. It seems already apparent that in dealing with strictly neurotropic agents we are confronted with practical immunologic difficulties not encountered in generalized virus infections or in bacterial disease.

Whatever the virus agents may be, there is no doubt about the fact that they are antigenic in the broad sense of the word. There are practically no exceptions to the rule that spontaneous infection with a virus produces some sort of a specific immunity, though the solidity and permanence of such immunity varies.

In smallpox immunity is usually for life, although second attacks may occur from one in two hundred, to one in fifty thousand, according to statistics of different observers. Taking the extremes of observation, the immunity may last from two to sixty years.

In measles, second attacks occur but rarely. In mumps immunity is usually for life. In poliomyelitis, a second attack is almost unheard of.

Immunity for life usually follows varicella, yellow fever, fowl plague, cattle plague, hog cholera, and canine distemper. In foot-and-mouth disease, immunity lasts about two years: in cattle and guinea pigs, about a year.

In dengue, on the other hand, immunity, according to Siler, may be as short-lived as fifty-three days. It is short in duration in Pappataci fever.

In herpes in man there seems to be practically no local immunity; second attacks may even occur habitually at the site of preceding lesions; yet, in corneal herpes of rabbits we have found that immunity to subsequent intracerebral inoculation develops and may last for months. This, probably, depends upon special circumstances. Most people who

habitually develop herpes of the lips or elsewhere after fever, X ray, sunlight, vaccine injections, etc., have a general immunity in the form of neutralizing bodies in the blood and in such people intraspinal injection of herpes virus has given negative results. It is probable that the herpes virus, like other filterable agents, may remain latent in the body in special locations, often at the mucocutaneous junctions and become locally active in spite of general immunity, as a result of damage to cells in the neighborhood of its habitual lodgment.

There has been much discussion concerning the dependence of immunity upon persistence of the virus in the body after recovery. De Kock (6) observed a case of equine pernicious anemia in which the blood of the horse remained infectious for seven years after the attack. Bang (7) has reported the infectiousness of a bull two and a half years after recovery from an attack of foot-and-mouth disease, and Olitsky, Traum, and Schoening (8) recovered the infectious material from the healed lesions on the hoof of an animal thirty-four days after infection. In poliomyelitis the virus has been found by Lucas and Osgood * in the nasal secretions of a child four months after the second attack of the disease. Again Cole and Kuttner (11) have found that the virus of the salivary glands of guinea pigs can be recovered throughout the period of immunity. Lipschütz (12) has found the same thing in contagious epithelioma of fowl. Theiler (13) reports similar observations in African horse sickness, and Rivers and Pearce (14) have found "Virus 3" in a transplanted neoplasm of a rabbit and in metastases, the animal having become immune to the virus in the meantime. Noguchi (15, 16) found vaccinia virus in a rabbit twenty-two days after the establishment of cutaneous immunity and Douglas, Smith, and Price (17) found the virus in the organs of rabbits forty-one days after inoculation. Many similar observations have been collected and reported by Winkler (18). Persistence of virus in immune animals has been demonstrated by Olitsky and Long (19), who, by cataphoresis, recovered vaccinia virus from tissue suspensions negative by the usual animal inoculation tests, and subjected to experiment long after the animals had recovered. Of similar significance is the later extension of the work of Rivers and Pearce (20), who found that virus III could be carried from rabbit to rabbit, together with rabbit carcinoma. Theiler (21) obtained the virus of the spontaneous encephalitis of mice from the cords of these animals almost a year after recovery. It appears not at all impossible that the principle may be more widely applicable than hitherto supposed. Even in bacterial diseases such as hemolytic streptococcus infections, the latent presence

* The observations of Lucas and Osgood (9, 10) probably have merely the significance of indicating a "carrier" state analogous to that prevailing in many bacterial diseases.

and complete inactivity of the organisms in human tissues have been demonstrated by bacteriological study of secondary repair operations, and our own statistical studies in Brill's (22) disease showed quite definitely that the Rickettsiae may remain latent for many years in a percentage of individuals infected in youth.

Virus agents are distinctly antigenic in the sense that they produce specific reaction bodies which may be experimentally demonstrated in the sera of experimentally or spontaneously infected individuals. Their presence lasts for varying periods. In some cases — as for instance spontaneous psittacosis — they appear in slight concentration and do not last long. At the other extreme is yellow fever, in which these reaction bodies probably persist for life and have actually been found longer than thirty-three years after the attack. The nature of the antigen of the virus agents is of course deeply bound up with the problem of the nature of the agents themselves. If crystallization studies like those of Stanley (2, 3) on mosaic disease are extended to other varieties of virus and if the inanimate protein nature of the active products is eventually proved, we would have to assume that the infected cells of the animal itself had either modified homologous cell protein or had synthetically by what Stanley calls a process of autocatalysis produced a protein substance which was chemically heterologous and therefore auto-antigenic. On the other hand, a recent study of the elementary bodies of vaccinia by Hughes, Parker, and Rivers (23) appears to demonstrate that the vaccine virus contains carbohydrate, fat, and nitrogen, the last apparently in the form of a protein. The analogy with bacteria is striking, but until tissue culture methods improve and extend quantitatively, no precise information can be expected.

The crystallization of the tobacco mosaic virus by Stanley has furnished an opportunity for immunologic work with what may be regarded as the chemically purest form in which any virus has been so far produced. Chester (24), who has done such tests with Stanley's preparations, has adduced evidence which leaves little question regarding the antigenicity of a virus separate from accompanying inactive protein. Neutralization studies with other varieties of virus have, of course, shown the same thing in principle. But Chester's results, because of the nature of his materials, have removed all possible doubt that, although the virus agents depend upon the metabolic activity of the protein of the host cells, they represent an antigenically individual substance, serologically differentiable from the proteins of the host. His conclusions may be summarized as follows:

Purified tobacco mosaic virus retains its ability to react with immune serum produced either with whole sap or with purified virus. The precipitogenic power of the virus remains constant throughout such purification.

Fractionation of tobacco mosaic extract by filtration through graded membranes shows that the precipitating power is retained in the fraction that contains the virulent material.

When the same virus is propagated in species of hosts widely separated taxonomically, the common serologic reaction is retained and, regardless of species, still varies in strength proportionately to the amount of infectious material present.

The common method of demonstrating the presence of the "protective bodies" depends upon mixing immune serum and measured doses of virus in test tubes and injecting these mixtures into susceptible animals. What is the mechanism of the neutralization? Rivers and his associates showed that normal cornea inoculated with vaccinia virus and then cultivated in anti-vaccinal plasma nevertheless developed typical lesions, and analogous studies with virus III carried out by Andrewes (25, 26) showed conversely that if immune serum were added to the cells before the addition of the virus, infection was prevented. If, on the other hand, virus and cells were first brought together and serum then added, infection took its ordinary course. Again, when immune serum was injected into the skin before or together with vaccinia virus, no lesion resulted, but if the serum followed the virus even in as short a time as five minutes, lesions were not prevented.

There is evidence to indicate that there is no stable union between virus and antibody such as that which apparently takes place in analogous bacterial reactions, and that the antibody does not actually kill the virus.* In 1931 Schultz (27) showed that when poliomyelitis virus and undiluted immune monkey serum were incubated together for two hours and then injected into monkeys, the virus was neutralized. When, however, such mixtures were diluted with nine volumes of salt solution and proportionate amounts injected into a monkey, poliomyelitis resulted. At almost the same time, Andrewes showed that virus could be recovered from negative mixtures not only by simple dilution, but by absorption on kaolin or simply by allowing plasma to clot in the mixtures. That tissue susceptibility influences such experiments is indicated by the demonstration that mixtures of immune serum and vaccinia, though neutral for the skin, are still infectious for the brain and testes or by way of the circulation, even when the amounts of virus lay within the minimal infective doses for the brain. In a similar manner, Todd (28, 29, 30) showed that fowl plague virus and immune serum might be infectious intravenously though negative intramuscularly.

As far as the dilution experiments are concerned it is not unlikely that

* We would like to interpolate that alexin studies should be undertaken in this connection because Mueller's work on Rous sarcoma suggested that the presence or absence of alexin strongly influenced protection experiments.

the distinction between virus antibodies and bacterial antibodies may be more fictitious than real. Enders and Shaffer (31) have carried out experiments with pneumococcus and pneumococcus antibodies which, both in principle and results, are entirely analogous to the virus-antibody "dilution" phenomena of Schultz and of Andrewes.

Sabin (32, 33) has added further evidence to the conception that virus anti-substances do not actually combine with the virus or form a loose combination only. When active Berkefeld "V" filtrates of vaccinia virus were incubated with an excess of immune serum for a number of days and then centrifugalized for three hours at 14,000 r.p.m., the sedimented virus remained fully infectious. In this case, the addition of alexin made no difference. Further, when serum with an excess of virus was incubated at 37° C. for three hours or for twenty-four hours at room temperature and then put through a Seitz filter that held back the virus, the filtrate was as quantitatively protective as the original serum. An analogous experiment was done with the virus of herpes and its antiserum.

Experiments with vaccinia have been particularly instructive. When vaccinia cultures are made with normal serum, the virus attaches itself to the tissues promptly and multiplies. When cultures are set up in immune serum, the vaccinia becomes similarly attached to the tissues but does not multiply. If such infected tissue plus immune serum is then washed until the washings show no further protective bodies in test, the tissue remains refractory, but is infectious for other tissues. Profuse washings of tissue-immune-serum combinations render the tissue again susceptible.

From all this we may conclude that the so-called "antibodies" or protective bodies against viruses do not form stable compounds with the virus and they do not kill it. They seem to interpose themselves between the susceptible cells and the virus. We are dealing, then, with conditions in which protective bodies and infectious agent may coexist side by side in the presence of susceptible tissues, and in which infection or protection depend not only on the presence of both substances but upon the sequence in which they establish contact with the point of attack. All these considerations must be included in any theoretical conception or practical efforts which concern themselves with virus immunity, and should especially influence clinical views on the therapeutic use of antiserum. They should make us hopeful of the prophylactic possibilities of virus protective sera, but correspondingly skeptical of curative claims. Much money, effort, and heartache could have been saved if these experimental facts had been considered before wholesale treatment with convalescent serum was exploited. On the other hand, we think it premature to conclude that virus antibodies differ fundamentally from those

formed in defense against bacteria. Many of the phenomena discussed above for virus antibodies find analogies in the study of bacterial antibodies and the physical differences imposed by the minute size, and consequently large surface exposure of the virus agents, might well explain many of these differences. Moreover the fact that virus infection is primarily an intracellular process should be considered in all practical applications of immunological principles.

The presence of antibodies against virus infection has usually been determined by protection reactions in which virus is mixed in suitable dosage with antiserum, the mixtures allowed to stand, and then injected into susceptible animals by the suitable route. *In vitro*, reactions of virus antigens analogous to those obtained in bacterial infections and immunizations have, however, been successfully carried out by a number of observers. Gordon (34) obtained specific alexin fixation and precipitation by mixing immune serum with concentrated preparations of vaccinia and smallpox virus. Burgess, Craigie, and Tulloch (35), with the same materials, observed precipitin reactions. In foot-and-mouth disease Ciuca (36) reported alexin fixation. In many of the earlier investigations of this kind there was some question about the specificity of such reactions. Schultz and his co-workers (37, 38, 39, 40) suggested that contaminating bacteria in the virus antigens might be responsible for the reactions reported. However, the carefully controlled experiments of Bedson and Bland (41) have shown beyond question that specific alexin fixation can be obtained with the virus agents of vaccinia and herpes and their respective hyperimmune sera produced in guinea pigs. Similar experiments have been carried out in yellow fever, and Francis (42) has obtained valid alexin fixation reactions with epidemic influenza virus and the sera of convalescent ferrets and human beings. That specific antigenic reactions have been obtained by Chester (24, 43, 44) with Stanley's crystallized tobacco mosaic virus has been mentioned.

Virus agents, then, like other protein complexes, have specific antigenic properties, and it is not at all unlikely that observed differences between virus-antibody reactions and those between other antigens and their antibodies, depend not upon any difference in immunological principle, but rather upon the small size and great surface exposure of the antigenic material and, *in vivo*, upon the intracellular position of the virus agents in the diseased animal body.

Artificial Active Immunization. Since immunity to spontaneous infection is a rule in virus disease as regularly as in bacterial infections, one has naturally followed the analogies of bacterial investigation in efforts to protect. Such investigations have revealed the fact that it is either impossible or extremely difficult to immunize against any virus

infection with killed virus. All the methods which up to the present have become incontestably established as effective in virus immunization have depended upon the employment of specifically altered or attenuated virus, living virus used in extremely small amounts, or serum-virus combinations. It is worth noting, for the sake of emphasizing the analogy with certain bacterial problems, that similar difficulties exist in regard to immunization with some bacteria. It has so far been impossible to produce a solid immunity against anthrax with killed cultures even when, as in experiments of our own, the vaccines were made of formalinized virulent anthrax bacilli obtained directly from the peritoneal cavities and organs of anthrax-killed animals. Similarly, it is so far impossible to produce a solid active immunity with killed "M" dissociants of the hemolytic streptococci. It is important to remember, therefore, that here again the problems of the two types of infection are closely related.

The most notable exceptions to the rule that killed virus does not protect concern particularly rabies and canine distemper. In regard to rabies, the problem is at present unanswerable. There are experimental difficulties in infecting animals with rabies with regularity by any but the intracerebral route, which impede precise experimentation. The intraperitoneal mouse technique now being investigated by Webster (45) shows promise of eventually throwing more light on this difficult problem. It is not easy to demonstrate conclusively whether the rabies virus in the phenolized Semple preparations at any given time is dead, attenuated, or diminished in amount. We have seen a case of rabies, thoroughly and promptly treated with the full series of phenolized rabies virus injections, which remained utterly unprotected, dying of rabies after a short incubation time. We have found in the laboratory that living virus can be demonstrated for 48 hours at least in preparations treated with phenol by the Semple method. Kelser has put Semple preparations through a fine copper screen and found surviving virus for a number of days in the little clumps that remained on top of the screen; and a year ago Kelser, in our laboratory, recovered *virus fixé* from the brain of a dog paralyzed after an injection of supposedly killed rabies virus. We are thus dealing with preparations in which the margin between survival and death requires considerable additional investigation. The recent experiments of Webster and Dawson, who found viricidal substances in the serum of Semple-treated individuals, indicate that certain of these preparations are active, but whether or not this is due to the residual presence of living virus cannot yet be decided.

Similar uncertainty exists regarding the canine distemper immunizations of Laidlaw and Dunkin (46, 47, 48). From the extreme precision with which the formalin treatment of the animal spleen material must be

adjusted for successful immunization, one is inclined to suspect here, also, the possibility of attenuation or of the quantitative reduction of living virus. Moreover, inasmuch as spontaneous immunity in this disease appears to be permanent and the artificial immunity is short-lived unless subsequently fortified with active virus, it is apparent that an important difference exists between the immunizing properties of formalinized preparations and those of the living agents. Dunkin (49) himself says: "There seems to be a stage in the degradation of the virus when it will immunize without giving disease," and he adds that this critical period is of short duration — an observation which is entirely consistent with the assumption of attenuation rather than killing. Another and perhaps fundamental observation made by these investigators is the following: They found that vaccine made from formalinized dog spleens was more effective than that made of ferret's spleens for the immunization of dogs and vice versa. This observation is not easily explained by ordinary immunological reasoning, but must be remembered for correlation with future observations. It is possible that a small amount of living virus adapted to the dog and relatively less infectious for the ferret might still suffice to incite mild infection in the species to which it is adapted and therefore immunize, but be insufficient to become active in an animal for which it is relatively less virulent. And as we have seen, virus agents are far more flexible in their species adaptations than are the bacteria. Moreover, this observation, though unexplained, tends to strengthen the conception that virus agents are autocatalytically produced from the proteins of the host.

In studying herpes immunity, Tang (50) and the writer have found that no immunity whatever was obtained when herpes virus was inoculated either in amounts so small or in serum mixtures so neutralized that it aroused no reactions in the animals. Some indication of infection, if only a mild fever, appeared to be necessary for the development of immunity. Similarly, negative results have been recently obtained by McKinley and Thompson (51) with herpes virus killed by heat and inoculated in large amounts. However, we tested for immunity by intracerebral inoculation and McKinley and Thompson used the intraspinous injection — methods far too drastic for the appraisal of a partial immunity, both involving the complex problem of relationship between generalized immunity and local susceptibility of the nervous system. (See Benson's work below.)

It has been a question in the minds of immunologists whether or not the immunizing effectiveness of killed virus agents might not be largely a quantitative problem. We may cite in analogy our experiences with typhus fever. It was formerly supposed that typhus immunity could be produced only with living virus. Yet when we had developed a

method which made it possible to inject enormous amounts of killed Rickettsiae — amounts quantitatively comparable to those injected, for instance, in typhoid vaccination — immunity could be obtained. An indication that the quantitative considerations may play a similar role in virus immunity is furnished by a number of studies. Gordon (34), working with vaccinia, found that this virus, heated to 55° for 30 minutes, still possessed protective power, but not unless very large quantities were used. Parker and Rivers subjected vaccinia virus to 0.3 per cent formalin and found that the inactive elementary bodies so treated were still capable of producing protective bodies in rabbits. Their results were achieved by six weeks' courses of injections given on two successive days of each week, with increasing amounts of the inactivated virus. The preservation of antigenic properties by formalin, even when the formalinized preparations were subsequently heated, appears from the work of Bedson (52, 53) with the agents of herpes and psittacosis. It is not improbable that the divergence of his results with herpes from our own and from those obtained by McKinley and Thompson is due to the fact that instead of working with neurotropic infection in rabbits, he studied a herpes virus adapted to the footpads of guinea pigs — a method far more delicate and not involving the peculiar problems concerning the nervous system. He obtained a demonstrable immunity by the injection of the virus agent treated with 0.2 per cent formalin, even when the formalinized material was steamed for 20 minutes after the addition of the formalin. When steaming alone was used, the immunizing power was destroyed.

It is therefore not out of question that a demonstrable degree of immunization may be obtained with killed virus when tissue culture methods, and possibly other techniques, have enabled us to obtain high concentrations of the virus agents; and from the practical point of view such material may serve, if not for full immunization, at least for vaccination preparatory to the subsequent administration of small amounts of living virus or partially neutralized sero-vaccine. Indeed, such a method appears to be gaining headway in connection with canine distemper. That generalization is again unwise follows from certain experiments of Aycock (54), who has found that even repeated subcutaneous and cutaneous inoculations of living poliomyelitis virus do not necessarily protect against subsequent nasal instillation of fully virulent material, even though the preliminary vaccinations may lead to a varying percentage of fatal infection. This must be regarded as a special case involving the relationship of general immunity to neurotropic infection.

Meanwhile, the problem of solid immunization with killed virus agents is closely related to that already discussed regarding the survival of these

agents in the naturally immune animal. Certainly the incomplete results so far achieved with killed virus tend to fortify the view that prolonged immunity is in some way related to such survival.

The matter of sero-vaccination involves special problems. From what we have said in the discussion of protective bodies it follows that the immune serum neither goes into irreversible union with nor kills the infectious agent. As we have seen, Sabin has shown that in the presence of immune serum a virus agent may become attached to the tissues without multiplication, but when such tissue is washed until the washings show no further protective bodies, this tissue remains refractory but may be infectious for other tissues. It is on some such basis that one could explain the successful sero-vaccination against yellow fever carried out by Sawyer and his associates. It is true that our own results with herpes showed that completely neutralized mixtures had no immunizing power, but these experiments should be repeated with dermal reinoculations rather than with intracerebral or intraspinous methods by a technique something like that of Bedson.

In conclusion it may be said that investigations with immunization in filterable virus infections have gone far enough to indicate clearly directions of effort. We may summarize these as follows:

1. Attempts to produce concentrations of the individual agents by tissue culture or by the inoculation of animals with artificially increased susceptibilities to permit further quantitative study of the immunization with killed viruses. In such efforts the killing agent of choice so far appears to be formalin.

2. The further investigation of serum-vaccine mixtures with subsequent tests of immunity not involving direct injections of the nervous system but simulating spontaneous paths of infection.

3. Study of the relationship of general immunity to direct neurotropic inoculation, with greater precision of quantitative measurements.

In connection with the last problem, it appears entirely premature to carry out large-scale human vaccinations against such diseases as poliomyelitis which, in the absence of further information, are probably useless when the administered virus is killed, or dangerous when it is supposedly attenuated.

Immunity in the So-called "Rickettsia Diseases." (European and Mexican typhus fever, spotted fever, fièvre boutonneuse, tsutsugamushi disease, trench fever.) In the group of diseases which are caused by the organisms spoken of as Rickettsia, immunological conditions differ in a number of important respects from those prevailing in bacterial diseases. All of these diseases leave behind them an immunity which lasts a long time, although individual instances of second attacks

which sometimes have been fatal have been recorded two years and later after the first infection. Trench fever, about which relatively little is known, seems to differ from the others in that within the first few years after the original disease febrile relapses are not uncommon. This can only be interpreted as meaning that the virus remains latent in the body and becomes active as the immunity diminishes. In the Brill's type of the classical European disease our statistical studies have indicated that this actually occurs.

The conditions in the Rickettsia diseases are probably different from those in the bacterial infections largely because the organisms, though circulating in the blood during the early febrile stages, later concentrate intracellularly in the vascular endothelium and perhaps in the cells lining serous cavities.

Up to quite recently it was assumed that in typhus fever only an attack could immunize, and practically all experimentation aimed at active immunization was done with supposedly attenuated virus. Thus Nicolle, Sparrow, and Conseil (55) had promising results by inoculating a number of human beings with the serum of infectious guinea pig blood, such serum being relatively less infectious for man than human blood taken in a similar way. Similarly encouraging results were obtained by repeating inoculations of minute doses of brain, quantities ranging from 1/5000th to 1/10,000th of a brain.

Guinea pigs which have withstood an attack and completed the temperature curve characteristic of this infection are thereafter immune. As soon as the temperature has returned to normal, the serum of such animals, mixed with virus before injection, protects. Such protective effect is established immediately after the completion of the disease but rarely remains effective longer than two to three weeks. The same thing has been shown for human convalescent serum by Nicolle and Conseil (56), who have suggested the use of such serum as a prophylactic measure and believe that, for a short time, the administration of such serum will protect. The short period during which the serum of recovered cases remains protective suggests that these antityphus protective bodies are not in every way comparable to true antibodies.

Attempts to produce active immunization in man by injecting virulent blood inactivated by heating to 60° C. were first tried on troops of the Turkish Army by Hamdi (57) in 1916, and were thoroughly investigated, with questionable results, upon German troops in Roumania by Otto and his associates (58). Somewhat later, Nicolle (59) endeavored to develop methods of active immunization in man by the injection of minimal infectious doses of virulent guinea pig material. This method has been further developed, particularly by Blanc (60), but involves a certain amount of danger, an opinion supported by the disastrous con-

sequences which followed the use of supposedly attenuated guinea pig virus in recent vaccinations in Chile.

The use of fully virulent material for vaccination in an infectious disease as dangerous as typhus fever is never justified as long as there remains any possibility of achieving immunization with killed virus, or as long as reliable and constant methods of attenuation are available. Our own experience with typhus virus in the form of virulent blood or tissues has convinced us that there exists at present no reliable method for controlled attenuation. Either such material is virulent, and consequently dangerous, or else it is killed and has no immunization value because of the small number of Rickettsiae present.

The first investigations which established the possibility that immunity could be achieved in Rickettsia diseases with a killed virus were those of Spencer and Parker (61) with Rocky Mountain spotted fever. Their vaccine was made with potent suspensions of the viscera of infected ticks, ground in a mortar and killed with 0.5 per cent phenol. With such vaccine, they succeeded in protecting guinea pigs and monkeys, and in man the results, so far as can be judged at present, indicate either complete prevention or a modification of subsequent infection in the direction of great mildness. A method similarly conceived was applied by Weigl (62) to the classical typhus. Weigl used an ingenious method of rectal infection of lice, and made his vaccine from the suspensions of infected louse intestines. The Weigl method is undoubtedly as effective as any method so far devised. However, the rectal injection of lice, although not excessively difficult, is still a laborious process, and the insects, once inoculated, must be fed for over a week on immune individuals before they yield an adequate harvest of Rickettsiae. Moreover, the immunization of a single individual requires the yield of 100 lice or more. For these reasons, the Weigl method cannot be regarded as a practical solution of the problem of large-scale prophylaxis.

In 1931, with Batchelder (63), the writer found that guinea pigs could be immunized with formalinized suspensions of heavily infected murine guinea pig tunica. Basing subsequent efforts upon this fact, methods have now been developed for the production of large quantities of murine Rickettsiae in X-rayed rats (64). The thick suspensions so obtained are formalinized and have been shown to protect guinea pigs against typhus infection. With such vaccines, large-scale human immunization has been carried out in Mexico. We have also immunized horses with these vaccines, thus producing a horse serum which has powerful protective effects against murine infection, and definite, though less complete protective potency against the related classical virus. The serum has also been used for short-lived prophylaxis in village epidemics in Mexico by Bustamante and Varela (65). We abstain from further discussion

of these methods, which are being tested in man, since they have been published in detail.

We consider that the murine methods do not completely solve the European problem because of the incomplete protective potency against this infection of both the murine vaccines and the anti-murine horse serum. And since, with the classical virus, we are unable to produce the large yields of Rickettsiae in X-rayed rats, we have turned to two other methods with this virus.

One of these is a quantitatively augmented tissue culture procedure based in principle on the method first developed by Nigg and Landsteiner (66). With vaccine so produced, Macchiavello-Varas and the writer (67) have confirmed the publication of Kligler and Aschner (68), in showing that formalinized tissue culture Rickettsiae would protect guinea pigs. For large scale practical purposes, this method is not yet sufficiently developed for routine application, but the results which have already been achieved indicate that improvement of technique and organization of tissue culture production may render this procedure applicable on a practical scale.

The other method which, with Macchiavello-Varas, we have shown to hold some promise of practical usefulness, particularly in the face of epidemic conditions where vaccine production by the other methods might lag behind quantitative needs, consists of a sero-vaccination in which virus in the form of infectious guinea pig blood or of tissue culture is injected together with protective convalescent guinea pig serum, or the anti-murine horse serum. It is possible to achieve active immunization in this way, without any reaction to the sero-vaccination, when the balance between the two reagents is accurately adjusted. When the virus is not completely neutralized by the serum, slight and temporary febrile reactions follow the vaccination.

Both methods are ready for study upon man — the former under ordinary circumstances of small, local epidemic; the sero-vaccination, which may still involve a certain degree of risk, in epidemic emergency.

Immunity in Protozoan Infections. The field of protozoology is so large and varied that it is quite impossible to summarize the immunological reactions which occur in the many diseases caused by forms differing in invasion, localizations, and pathological reactions. The immunology of protozoan infections has been comprehensively reviewed by Talliaferro (69) and by Schilling (70) and is dealt with in various monographs on the individual diseases. We can do no more than outline briefly the condition in which the main protozoan diseases show analogy to or differences from bacterial and other infections in immunological principles.

As a general rule the protozoan invaders are more limitedly specific

than are most of the bacterial infections, in this resembling more closely some of the filterable virus diseases and the conditions produced by treponemata.

Immunity after a first attack in the protozoan infections, in the sense that the increased resistance lasts after the organisms have completely disappeared is relatively rare. One of these is Leishmania infection, the so-called Oriental sore in which in a good many cases immunity against the development of a second sore results from a primary infection. To this, Talliaferro states, there are many exceptions and adds that the immunity results only when an experimentally conferred sore is allowed to run its natural course. By means of immunity tests, Laveran (71) has shown a relationship between the Mediterranean kala-azar and the Indian variety, since a monkey inoculated with the former was immune to the latter.

In malaria the question of immunity has been much investigated. In bird malaria the conditions are similar to those described for syphilis in man in that immunity lasts just so long as the body still harbors the living parasites. In untreated malarial patients, in the course of years, the relapses are apt to be less prolonged and less severe, and the inhabitants of tropical countries infected in childhood are less subject in adult life. The investigation of malarial immunity gained much in accuracy by the development of the method of treating paresis with malarial blood. It has been found in a number of cases (Nicole and Steel) (72, 73) that after a first successful injection of malarial blood it was occasionally difficult to produce a second attack in the same way. Talliaferro states that infection with repeated inoculations by mosquitoes confers relative immunity to acute symptoms, but that there is little or no evidence that acquired resistance persists for any length of time after complete recovery.

In piroplasmosis, Smith and Kilborne (74, 75) as early as 1893 reported that one attack of Texas fever conferred immunity, but it was also shown that such immunity was associated with persistent harboring of the organisms. Schroeder and Cotton (76) reported a case in which the organisms were present for twelve years. In the piroplasmosis of horses, Theiler (77) has noted similar conditions. He showed that fatal disease could be produced with the blood of a horse that had survived an acute attack two years before.

In the trypanosome diseases, immunological studies have been carried out with non-pathogenic *Trypanosoma lewisi* of rats with which Kanthack, Durham, and Blandford (78) showed that a rat which has recovered does not *take* on second inoculation. Schilling (79) questions the persistence of immunity after disappearance of trypanosomes even in this case, since he found that the organisms may remain for many

months in the body of the animals, though they may not appear in the blood, and since, as a general rule, the immunity is over in three months or slightly longer.

In amebic dysentery as far as we know no definite immunity is established and the parasites persist for many years, though acute intestinal symptoms may be absent.

Evidence of Antibodies. Immunity in the protozoa is often associated with specific antisubstances in the blood. Animals with experimental trypanosomiasis often develop trypanolytic properties in the serum which is capable of killing the parasites. This limits the number of invading organisms, drives them out of the blood, and converts the active disease into a latent one. In such animals Talliaferro (80, 81) has found reproduction-inhibiting properties in the serum which limit cleavage of the organisms. In *Trypanosoma lewisi* infections this was apparent as early as the tenth day.

In malaria the conditions in the variety existing in birds have been analyzed by L. G. Talliaferro (82) as follows:

"During the first part of the infection relatively few parasites are killed, so that they accumulate in the blood and give rise to the acute stage. Sooner or later, however, a large proportion of the parasites are killed and there may even be a temperature relapse, but eventually destruction equalizes or exceeds the number produced. In time the destruction becomes so great that no parasites can be found in the blood, but their presence can be demonstrated by Whitmore's technique by injecting blood into uninfected canaries."

L. G. and W. H. Talliaferro (83) have been unable to demonstrate that the removal of the parasites is dependent upon antibodies. There is, according to these writers, no proof that the phagocytic mechanism is enhanced. This opinion is shared by Schilling (84).

Agglomeration of trypanosomata analogous to bacterial agglutination has been described by Laveran and Mesnil (85, 86). This has been particularly studied with *Trypanosoma lewisi* but has also been worked out with other trypanosomata. Schilling and Jaffé (87) believe that these reactions are relatively specific. Laveran and Mesnil have also noted an opsonin-like action consisting in rapid phagocytosis when various trypanosomes were injected together with specific anti-serum into the peritoneal cavities of guinea pigs.

Alexin fixation has been carried out by Landsteiner, Müller, and Pötzl (88), who used an antigen made with the liver of guinea pigs infected with *Trypanosoma equiperdum;* the serum was that of a rabbit infected with this organism. With Leishmania, Noguchi and Kligler (89) produced potent agglutinating sera in rabbits.

In amebic dysentery, many attempts have been made to utilize serum

reactions for diagnosis. Craig (90) in 1927, using alcoholic extracts of cultures of *endameba histolytica* as antigen, obtained alexin fixation with the sera of individuals infected with the parasite. He described a technique by which he was able to obtain positive reactions in 92 per cent of 84 cases in which the organisms could be found in the feces (91, 92).

A curious serological reaction with protozoa is the "adhesion phenomenon" described by Rieckenberg (93). Working with *trypanosoma brucei*, he mixed citrated blood of recovered rats with the organisms *in vitro* and found that masses of blood platelets adhered to the trypanosomes. He found that the reaction was specific for individual strains and used it for differential purposes. Kritschewski and Tscherikower (94, 95, 96) found that the reaction was not a specific blood platelet one, for they could replace the rat blood platelets with those of normal animals. They believed the reaction to be dependent upon fibrinogen, since it did not occur in serum from clotted blood, but only with citrated plasma. They also found that this peculiar antibody could be produced by the injection of dead trypanosomes. Subsequently Krantz (97) found that the part played by the platelets was a purely mechanical one and that other particles — bacteria, for instance — could serve as indicators equally well. Davis and Brown (98, 99) have confirmed these observations with trypanosomes and with leptospira icterohemorrhagiae, and believe that the reaction is sufficiently specific to be diagnostically applicable. They assert that the antibody is present not only in the plasma but also in the serum and withstands a temperature of 65° C. for two hours.

As far as one can summarize, a relative immunity and, in some cases, a considerable immunity appears to be established in most protozoan infections after an attack of the disease, but, except in a few instances, this immunity is of the nature of a resistance to super-infection rather than one of true immunity. In the majority of the conditions in which relative resistance persists, the organisms are still latent within the body.

Suppression of the protozoan invaders from the circulating blood and their limitation to circumscribed areas in the body seems in many cases to be dependent upon the development of specific serum constituents analogous to if not identical with bacterial antibodies. These are manifest in many conditions as parasiticidal substances, in some cases enhancing phagocytosis but in others merely limiting reproduction.

Serum reactions, from the diagnostic point of view, are beginning to have a certain amount of practical importance in some of the diseases, but as a rule have not been of more than theoretical value.

Simultaneous injection of specific sera with the parasites may prevent infection in animal experiments, but passive immunization with such sera in diseases of man has not, so far, given useful results.

Immunization with killed parasites or blood and organ material containing parasites has in general been unsuccessful. Immunization with attenuated organisms has given promise of useful application in a few diseases, but has not been sufficiently developed to be regarded as a method safe enough for practical application.*

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* The discussion of immunity to the protozoa is a most superficial one, partly because the subject is too extensive and partly because the subject is outside the immediate experience of the writers of this book. This is a special field which the reader will find ably treated in the monographs of Talliaferro and Schilling, from which we have freely quoted.

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CHAPTER XVIII

IMMUNITY IN SYPHILIS AND IN TUBERCULOSIS

THE immunological factors in these two great maladies are perhaps more obscure than in any of the infections. Because of the enormous importance of these diseases from the medical and economic standpoint, much effort has been expended in the attempt to reveal the mechanisms upon which depends the increased resistance which manifestly follows in most cases after the causative agents have entered the body. It should be stated at the beginning that humoral antibodies which do appear in tuberculosis, but of which even the existence is doubtful in syphilis, have never been shown to play a significant role. This fact at once compels us to postulate some sort of tissue immunity, which according to many authors persists no longer than the survival of the parasite within the body. In syphilis the conception of an immunity of infection, as we shall see, seems to accord best with the majority of clinical and experimental observations. But in tuberculosis an increasing body of testimony indicates that vaccination with the killed bacteria confers a certain degree of immunity albeit relatively slight and in all probability transitory.

In spite of the fact that it is now thirty odd years since Schaudinn identified *treponema pallidum* as the etiological agent, nearly all immunological studies in syphilis are still concerned with the single problem of whether or not resistance may endure after the parasites have been eliminated from the body. We know a little more about the manner in which the host checks, if it does not usually succeed in entirely overcoming, the attack of the tubercle bacillus. Since, unlike the treponema pallidum, this organism may be readily cultivated, it has been also possible to analyze the results of various modes of artificial immunization and to investigate the reactions of the tissues of normal and infected animals to the constituents of the bacterial cell. But even with this advantage we are as yet very far from being able to present a satisfactory description of immunity in tuberculosis.

Immunity in Syphilis

Evidence for the Development of Resistance Following Infection. It was stated in 1858 by Ricord (1) that "an individual who had once

acquired syphilis was thereafter protected against reinfection." This opinion acquired wide acceptance and was shared by most of his contemporaries. Bäumler (2) in 1875, summarizing the authoritative opinions of that period, stated that "One who has once had small-pox, scarlet fever, typhus, etc., is, as a rule, not liable to these diseases again for the rest of his life. The same is true of syphilis." However, even at the time Bäumler wrote this, exceptions to the supposed rule were accumulating and he appended, to the positive statement given above, references to observed instances of second infection reported by Bidenkap, H. Lee, Diday, Köber, Zeissl, and others.

The conception of the existence of acquired immunity was also expressed and widely accepted in the "laws" of Colles and of Profeta. The former, first enunciated by Beaumès and later, in 1837, stated by Abraham Colles (3), of Dublin, is a generalization based on the observation that mothers who have borne syphilitic infants were not infected by their children, although such children might infect wet nurses. Profeta's (4) observation was the converse of this, namely, that children born of mothers who suffered from active syphilis during the period of conception did not acquire the disease from their mothers.

Considering the intimate contact between mother and infant during the first months after birth, these observations acquired unusual importance. However, as we shall see, they have been deprived of much of their bearing as proofs of true acquired or inherited immunity by serological investigations such as those of Bauer (5) and Knöpfelmacher (6, 7) which have shown that mothers of syphilitic children usually give positive Wassermann reactions, a fact which makes it seem likely that such women are suffering from syphilis in a latent form and are not immune in the ordinary sense.

In earlier years much clinical research was carried out on reinoculation and what is commonly known as "superinfection" of syphilitic human beings with the object of demonstrating the development of immunity. Most of this work is unavailable as scientific evidence owing to the difficulties of distinguishing, at that time, between the true chancre and the chancroid, but some of the observations then made have been of much value in pointing out directions of later research upon animals. The work has been thoroughly reviewed and analyzed by Neisser (8) and by Levaditi (9). The records include both accidentally occurring superinfections and purposeful experimental reinoculations.

We may summarize the conclusions justified from a study of these observations:

1. The reports of Lasch (10), Jadassohn (11), Sabaréanu (12), Queyrat (13), Taylor (14), Knowles (15), and others, have shown that patients are susceptible to a second inoculation during the first incubation time, that is,

the period between the first infection and the appearance of the chancre. Second inoculations have also been successful at periods shortly subsequent to the appearance of the primary sore.

Autoinoculations and reinoculations undertaken after the chancre has become well developed have been, in the main, negative, though Queyrat reports a case successfully inoculated daily up to the eleventh, and Taylor, one inoculated on the fourteenth day after the appearance of the primary induration. The fact that many observers record failure is not of particular importance, since, after all, the appearance of the chancre does not mark off any fundamental change in the progressive pathological development of the disease, and indicates only the completed reaction at the point of entrance. The fact remains that analysis has revealed that reinoculation, with the appearance of a second lesion, is possible up to about the twentieth day after the appearance of the initial lesion.* It is stated by some of the observers that even when reinoculation during this period is successful, the incubation of the second and subsequent lesions is shorter, and that the induration itself is less severe and heals more readily than the first.

2. After the disease is well established as a systemic infection, that is, from the time of development of the chancre throughout the so-called active "secondary" period, reinoculation is either impossible or, at any rate, extremely difficult. Neisser cites Rollet (16) as follows:

"Although I and my predecessors have a thousand times attempted to reinoculate luetic subjects, we have never observed a successful case. I know no single fact more thoroughly proven than the insusceptibility of a syphilitic to the action of a new virus, and, moreover, these experiments are so harmless that they may be performed without scruple."

The same opinion was held by Mauriac (17) and is in a general way assented to by Neisser (8).

That the patient with well-developed lues has acquired a considerable degree of resistance to fresh inoculations is generally accepted, therefore, but there are investigators, notably Finger and Landsteiner (18), whose observations on patients with active syphilis indicate that superinfection is possible "under certain circumstances in all stages of the disease," but "the positive effect can be obtained only with considerable quantities of the virus." Furthermore, Landsteiner (19) states that lesions so obtained are relatively slight, do not appear as primary indurations, but simulate the particular variety of lesion manifest in the individual at the time. Were it not for Finger and Landsteiner's (20) monkey experiments, the simulation of the spontaneously occurring lesions by the inoculation-products would justify suspicion that these experimental results represented merely traumata in which, as points of less resistance, the patient's pre-existent disease had found a favorable spot for localization.

These observations are corroborated by the older literature. A report which has direct bearing on this point, is one of Queyrat and Pinade (21), who inoculated a tertiary patient with chancre material, obtaining not a primary sore but an ulcerated lesion having the clinical characteristics typical of the late skin manifestations of the disease. The autoinoculation experiments

* Mauriac stated the twenty-second day, and Neisser agreed with him. Taylor cited a case in which he succeeded in obtaining a second lesion with the patient's own virus on the fourteenth day, and Queyrat reinoculated with a positive result on the eleventh day.

of Ehrmann (22) also would tend to show that the resistance of the luetic subject is a relative one only. Ehrmann succeeded in producing positive autoinoculation products in 45 syphilitics with papular eruptions. Control inoculations with sterile water were negative. Although his observations and those of Finger and Landsteiner, with other similar ones, teach us that superinfection during the disease is possible, the nature of the lesions, their short incubation time, and their exceptional character when averaged with the total of such attempts, prevent them from invalidating the conclusion that there is a resistance at this stage higher than that of the normal subject.

We may conclude, therefore, that in the secondary period of the disease, the luetic individual has acquired a resistance which while not absolute is at least high, and protects him from fresh inoculation, although at the time his disease may still be progressing.

3. During the late stage of syphilis, the stage at which, according to the more or less arbitrary divisions of Ricord, we are accustomed to speak of it as "tertiary," the resistance is still manifest, though apparently not so regularly potent as during the preceding "secondary" period. Neisser expresses himself with great caution and accepts few, if any, of the observed reinoculations of tertiary cases as reliably representing freshly acquired infections. Nevertheless, he concludes that resistance during the late stages is pronounced but already beginning to wane. He himself in the Festschrift to F. Joseph Pick in 1898 cites a case of the development of a chancre in a tertiary case which was not followed by constitutional symptoms.

Clinical studies by Jonathan Hutchinson (23), Taylor (14), Hudélo (24), Neisser, and many others, have furnished data which throw much light upon the problem of reinfection. Of especial value is the painstaking analysis of reported cases of reinfection in syphilis made in 1909 by Felix John (25).

John takes the utmost care to separate the cases into the ones in which the evidence of true reinfection is absolute, and those in which the reported details are insufficient. In agreement with Taylor he insists upon a symptom-free interval of five years between the last manifestations of the first attack and the appearance of the second.

John has analyzed in this way 356 cases of supposed reinfection, in 34 of which the first attack was of congenital origin. Of the remaining 322, fourteen were cases which seemed unquestionable instances of reinfection and in 16 more there was practically no doubt of this. Of the 34 hereditary cases there were three, one of Emery, one of Taylor, and one of Hochsinger, in which there was practically no question of their nature as valid reinfections. In all of the others there was one point or another which rendered them doubtful as evidence.

John concludes that true reinfection can unquestionably occur in syphilis but that it is relatively rare.

To John's cases Neisser has added others reported between 1909 and 1911. Yet even with these, the total number is not a large one. Nevertheless, we should not be tempted to conclude from this that the relative infrequency of such cases is evidence in favor of the existence of a true

immunity analogous to that following typhoid, plague, etc. For we have seen that definite insusceptibility is coincident with the persistence of actual disease and, as Neisser points out, the investigations carried out with the aid of serological tests have shown that the number of cases uncured though long without symptoms, is much larger than formerly supposed. The scarcity of true reinfection, therefore, may well be due to the relative scarcity of completely cured cases.

Granted, then, that true reinfection is possible, is there any evidence that when it does occur, the second attack is less severe and more easily cured than the first, a fact which would also tend to support the opinion that a certain degree of immunity persists? Jonathan Hutchinson (23) expressed this view in a clinical lecture in which he states that "second chancres are far more common than second attacks of constitutional syphilis." However, John in his summary, in which Hutchinson's cases are included, finds that in general the disease has run a second course very similar as to severity to that incident to the first infection.

If we gather together, then, the facts revealed by clinical study they indicate:

1. The syphilitic subject acquires definite resistance to reinoculation, which becomes manifest soon after the appearance of the primary sore, when the virus may be regarded as having gained universal systemic distribution.

2. This resistance, high though not absolute, persists throughout the secondary or most active period of the disease and into the tertiary stage. During the latter, however, it appears somewhat to decline, reinoculation or superinfection being more frequently possible at this period.

3. When syphilis is entirely cured, susceptibility may in all probability be regarded as returning, possibly, though not certainly, to the same degree as it exists in the normal subject.

These are essentially the views enunciated by Neisser and upheld by perhaps the majority of observers, both clinical and experimental. But others, headed by Chesney (26, 27), have recorded much data in support of the hypothesis that the resistance acquired through an attack endures after a true cure has been effected. Practically all of this evidence is based upon work with animals — rabbits in particular — since the experimental and clinical observations on man such as those we have just summarized can be interpreted in support of Chesney's conception, as well as in the Neisserian sense. As far as we know, however, it is the opinion of most clinical syphilographers who have observed reinfections take the usual course in those cured by chemotherapy, that Neisser's dicta are probably correct. But let us examine a few of the facts which animal experimentation has contributed to the problem.

Susceptibility of Animals to Infection. In 1903, Metchnikoff and Roux (28) succeeded in transmitting the disease to a chimpanzee, and in March, 1905, Schaudinn (29) discovered the treponema pallidum.

Probable transmission of syphilis to lower monkeys had been accomplished as early as 1879 by Klebs (30) and subsequently by Martineau (31), but in none of these experiments had it been possible to prove the syphilitic nature of the inoculation products. In the chimpanzees inoculated by Metchnikoff and Roux, the animals developed not only primary sores, but secondary eruptions, polyadenitis, and enlarged spleens in characteristic manner.

Transmission to other anthropoids and to lower monkeys was then obtained by Metchnikoff and Roux, Ch. Nicolle, Neisser, Baermann and Halberstaedter, Finger and Landsteiner, Hoffman, and others. The susceptibility of monkeys was tabulated by Neisser in the following series: Chimpanzee, Gibbon, Orang-Outang, *Cynocephalus babuin*, *Cynocephalus sphinx*, *Cynocephalus hamadryas*, *Cercopithecus fulginosus*, *Macacus niger*, *M. nemestrinus*, *M. cynomolgus*, *M. sinicus*, *M. speciosus*, *M. rhesus*. In 1906, Bertarelli (32, 33) produced syphilitic keratitis in rabbits, demonstrating the *treponema pallidum* in sections of the cornea, and in 1907, Parodi (34) first produced syphilitic orchitis in the same animals.

Other species of animals, although undergoing an inapparent infection, seldom reveal any recognizable lesions and are therefore unsuitable for the ordinary type of experimental work. The production of keratitis has been reported in dogs and sheep by Bertarelli and Hoffman and Brünig, in guinea pigs by Bertarelli, in cats by Levaditi and Yamanouchi, and in goats by Bertarelli. Mice usually exhibit no symptoms or lesions, but become infected nevertheless (Uhlenhuth and Mulzer). Bessemans and DePotter (35, 36) have lately, however, observed the very exceptional occurrence of a chancre. Recently, Ch'uan-K'uei Hu and Pearce (37) have produced an inapparent infection in hamsters (*Cricetulus grisens*). Béclère (38) has claimed to have infected cattle and Worms (39) to have induced an inapparent infection in rats. A report of some years past on transmission to the llama has not been substantiated, but Van Haelst (40) in 1932 has confirmed Bertarelli's statement in regard to the susceptibility of the guinea pig and finds in this animal, in addition to primary lesions, a constant infection of the region lymph glands. The attempts of one of us (Zinsser) to infect cats, pigs, guinea pigs, rats, mice, and a few birds yielded only negative results.

Indications of Acquired Resistance in Susceptible Animals. The experiments on immunity to syphilis in apes and monkeys which were carried out by a number of workers following the discovery of Metchnikoff and Roux on the whole confirmed the observations concerning reinoculation and superinfection previously made on human beings.

The most extensive studies are those of Neisser (41). He reinoculated 135 monkeys 165 times with negative result on the second and subsequent

inoculations. The second inoculations were made at periods ranging from 21 days to two years after the first. In only 27 animals did the reinoculation show positive results, and in ten of these cases only does Neisser recognize the experiments as valid. Although these ten positive reinoculations, it is true, add an element of irregularity to the series, they constitute but 6.8 per cent of the entire number, a proportion which in no way invalidates the experiments when we consider that the work was done entirely on the lower monkeys, animals that are far less susceptible to syphilis than are human beings and in many of which, therefore, systemic distribution of the virus (a generalization apparently necessary for the development of resistance) may not have taken place. Neisser's conclusions, therefore, that monkeys, like human beings, are not reinoculable while suffering from systemic syphilis, seem entirely justified.

His work, as well as that of Finger and Landsteiner (20), and of Kraus and Volk (42) on lower monkeys, has shown that resistance does not develop until the twelfth to the twentieth or twenty-first day after the first inoculation, as in man. Finger and Landsteiner, furthermore, noticed that reinoculation-products, obtained by reinfection during the first incubation period, that is, before the development of the primary lesion, were less severe and developed in a shorter time than did the first lesion. This phenomenon, which would tend to mark another analogy to the conditions prevailing in human beings, was not observed in the experiments of Neisser and of Kraus and Volk. However, like the similar observation in human beings, it seems to indicate the gradual acquisition of resistance as the virus begins to exert its influence upon the tissues.

Again, with monkeys as in man, the question arises whether the resistance is a condition merely coexistent with active disease, or whether it may be interpreted as a true immunity which persists after the microorganisms have been completely removed. Investigating this question, Neisser reinoculated monkeys at periods ranging from 27 to 645 days after the first infection. After waiting a time sufficiently long to insure the negative result of the reinoculation, he used organ-substance from these animals to inoculate other monkeys. In 22 experiments of this kind he obtained positive results — showing that the organs of the apparently immune animals still harbored virulent treponemata. In seven animals only did the inoculations with organ-substance fail to produce lesions, but of these all but one died before the 30th day after inoculation.

In contrast with these results Neisser found that animals which had been "cured" by various treponemacidal agents such as atoxyl, arsacetin, etc., were almost regularly reinoculable. In fact, these experiments were so uniform that Neisser later utilized the reinoculation method as an index of cure or persistence of the disease.

The only other animals on which systematic experimentation has been possible up to the present time have been rabbits. Since Bertarelli's successful production of keratitis and Parodi's inoculation of

the testes in these animals, they have been studied carefully by a number of workers, among them Uhlenhuth and Mulzer (43), Noguchi (44), Pearce and Brown (45), Zinsser, Hopkins, and McBurney (46, 47), and Chesney * and his associates. The largest number of studies dealing with reinfection in rabbits are those in which in both instances testicular inoculation was practiced.

Truffi (49, 50) succeeded in obtaining a second infection in four animals, 12, 26, 76, and 514 days after the first.

Uhlenhuth and Mulzer (43) reinoculated eight animals in from 53 to 178 days and obtained successful reinoculation in seven of them.

In the experiments of Zinsser with Hopkins and McBurney (46, 47) seventeen syphilitic rabbits were reinoculated at intervals of from 38 to 458 days. In all but one the interval exceeded 100 days. Lesions containing treponemata were produced in three instances only and in five animals small nodules appeared following the second infection, but no treponemata could be found in the lesions. The strains were homologous in four instances and heterologous in thirteen, a matter to which we attached little importance at the time but which seems to have gained importance since then.

Brown and Pearce (51) inoculated rabbits with double syphilitic orchitis and reinoculated intracutaneously with the homologous strain 24 days after the first inoculation. Slight lesions were produced.

Kolle (52) in several hundred reinoculations found that before the 60th day, lesions are produced in about 60 per cent of the cases. After the 90th day no lesions develop.

The observations of Frei (53) are similar.

Reiter's (54) observations were similar to our own in that of nineteen rabbits reinoculated in from 133 to 602 days only three developed definite lesions.

Chesney and Kemp (55, 56) reinoculated eight rabbits with homologous strains in from 270 to 336 days after the first inoculations and obtained negative results throughout.

Chesney (26, 27), who has very thoroughly summarized the conditions described, concludes that rabbits establish a definite state of resistance towards a second infection with a homologous strain by the 90th day, and this resistance persists for at least four years. He believes that such resistance is more effective against homologous strains though it is effective in about 50 per cent of the cases in heterologous ones as well.

From these results it is apparent that a generalized resistance against testicular reinoculation develops in the rabbit following an initial

* The extensive studies of Chesney and his associates are brought together in his two reviews (26, 27), which have been cited. Reference may also be made to a more recent summary (48) of his views on immunity in syphilis in human beings.

infection in the same site. The findings are not so consistent, however, when the primary inoculation is intraocular. Bertarelli (33, 57) found that he could reinoculate the cornea in a rabbit that had previously been infected in the opposite eye, although this result was obtained in only one out of four animals. Purckhauer (58) and Uhlenhuth and Weidanz (59) found that a syphilitic keratitis of one eye does not protect against the corneal inoculation of the other eye. Uhlenhuth and Mulzer (43) and Neisser showed that infection of the eye could be produced while the opposite eye was still syphilitic. Tomasczewski (60) noted that scrotal infection did not protect against infection of the cornea and vice versa.

Truffi (49), on the other hand, after infecting one anterior chamber and both scrota obtained negative results upon inoculating after 147 days both anterior chambers and both scrota.

Frei (53), inoculating first by the testicular route and later intraocularly, obtained only 16 per cent positive results in 25 rabbits. The results obviously do not agree with those of Tomasczewski but compare with those of Adachi (61), who obtained five successful reinoculations in 18 rabbits prepared in the same manner as Frei's animals.

It is obvious that the evidence in regard to the resistance following corneal inoculation is conflicting. We may conclude, however, that in some cases rabbits with healed lesions of the eye are resistant to reinfection in the other eye, while others remain susceptible.

We may also state with reasonable certainty that corneal inoculation does not prevent successful reinfection of the scrotum. But when the reverse order of injection is effected, it would seem that a considerable resistance is induced to corneal reinfection by primary testicular inoculation.

There is suggested here that some degree of local tissue immunity or susceptibility may be involved. Indeed Ossola and Truffi affirmed that successful inoculation of the skin of rabbits confers a certain amount of skin resistance, and this is in harmony with the belief of Kraus and Volk that a specific skin immunity in syphilis is possible. In this connection the experiments of Zinsser, Hopkins, and McBurney (46, 47) are of interest. Twenty rabbits were reinoculated into the testes following primary unilateral, healed infections in these organs. It appeared that in rabbits the opposite testis can be successfully inoculated before, during, and after the existence of a testicular lesion on one side, but that reinoculation of the same testis which has apparently returned to the normal state, at periods ranging from six weeks to one year, is not often possible. Kolle (52) has confirmed and extended these observations and spoke of this local resistance as "chancre immunity," which he found is most complete when the same strain of treponema is used both for the first inoculation and for reinjection. If heterologous organisms are employed,

Kolle found that positive results upon reinoculation occurred in about 50 per cent of the cases. Chesney has also noted differences in strains of treponemata from human lesions and after passage in rabbits and emphasizes the necessity for recognizing this fact in interpreting experimental results.

From this summary of this work on the acquired resistance of man and animals to syphilis, it can be said that in untreated cases the response of the tissues becomes altered after the infection has progressed for a number of days, or until, as is often held, the organisms have generally invaded the body. This alteration is expressed by the failure of the tissues to react to a second infection by the usual pathological manifestations typical of the chancre. This state of "anergy," as Neisser has termed it, does not reach its maximum, at least in rabbits, until about 60 to 90 days after the primary inoculation, when it may persist for months and years, probably during the remainder of the host's existence. There is some ground for believing that it may be more intense in certain loci of the body than in others. The relative susceptibility of the cornea, at least in rabbits, seems to have been conclusively established, and Chesney (48) suggests that possibly the frequent recurrences of keratitis in congenital syphilitic children may be due to a similar lack of resistance of the eye in man. Finally there is much to indicate that this resistance of infection is more efficient when directed against the homologous strain than against strains of treponemata derived from other sources.

Two interesting problems remain for discussion. Is this resistance merely coeval with the residence of the treponema in the body, or may it persist after the organism has been eliminated by treatment with various antisyphilitic drugs? What is the mechanism of this resistance?

The Problem of the Persistence of Resistance Following Recovery. By use of arsphenamine and similar drugs at varying intervals after infection an animal can apparently be freed from treponemata, as determined by inoculation of samples of the various organs and body fluids into normal rabbits. In the case of rabbits Brown and Pearce (62) showed that treatment 18 days after infection renders the animal susceptible to infection 6 days later. Kolle (52, 63, 64), who has made valuable contributions to this problem, found that if syphilitic rabbits were similarly treated before the 45th to the 60th day of the disease, in many cases they became again susceptible to a second inoculation. On the other hand, if the disease were allowed to continue for 90 days, the animals usually became resistant in that they showed no lesion at the site of inoculation (chancre immunity). This condition persisted for a long time thereafter. Kolle interpreted his results as showing that abortive treatment was possible only when begun early in the disease, accepting Neisser's view that resistance to reinoculation is either a sign of the

persistence of the organisms or that an "inapparent infection," which he has shown may occur in rabbits, has been set up through the reinoculation.

Chesney's observations are entirely in agreement with those of Kolle in respect to the behavior of rabbits to reinfection following chemotherapy administered at different intervals, but he believes that even when treatment is given during the resistant period, the body is freed of organisms, and that in spite of this the resistance persists and the organisms introduced at the time of reinjection do not cause an inapparent type of infection but are destroyed. In support of his views Chesney and Kemp (55, 56, 65) showed that the lymph nodes of rabbits treated late in the disease were not infectious for normal animals. They then reinoculated these animals and failed to produce any indication of infection. They further controlled these results by demonstrating that samples of many of the organs of syphilitic rabbits similarly treated were non-infectious.

Kolle and Prigge (66) have criticized these experiments on the grounds that Chesney in some cases removed the organs when the drug was still present in active quantities, that he did not take into account the fact that the second inoculation might result in an inapparent infection, and finally that a "cure" had not really been obtained. The authors supported their contention by a large series of experiments from which they draw the conclusion that the treponemata of the second infection enter the lymphatic ganglia in spite of the fact that no lesion is produced at the site of the inoculation. Moreover, Worms (67) and Prigge (68) have observed that the lymph nodes removed soon after cessation of therapy may fail to be infectious, while those taken later after the drug has been entirely eliminated often produce typical lesions in normal rabbits. Bessemans (69, 70), who has lately reviewed the literature, is convinced by the work of Kolle and others, particularly as it reveals the possibility of inapparent infections both in man and in animals, that resistance is conditioned by and coterminous with infection. On the other hand, Uhlenhuth and Grossmann (71), Breinl (72), and others have attempted to support Chesney's hypothesis by numerous experiments. The objection raised by Kolle and Prigge concerning the persistence of treponemata following treatment, they have answered by showing that when large doses of salvarsan are administered during the period of resistance, it is impossible to demonstrate the presence of the organisms in the tissues, although Breinl admits that the complete sterilization of a syphilitic animal cannot be absolutely proved. He believes that the evidence, however, indicates that in many cases the body has been sterilized and that an immunity endures for some months which is sufficient to lead to the local destruction of treponemata at the site of injection.

The problem, then, at present remains unsolved but its ultimate solution would be highly desirable, since upon it depends the stimulus toward a renewed search for a biological prophylactic, as well as exceedingly important implications as to the possibility of complete cure of the infection.

Attempts to Demonstrate the Mechanism of Resistance in Syphilis. Immune Serum Reaction in Syphilis. The manner in which the body becomes comparatively resistant to the spirochete is entirely obscure. The Wassermann reaction at first seemed to signify that the serum of luetic patients developed specific antibodies to the invading microorganisms, but this view, of course, can no longer be maintained.*

When the apparent lack of specificity of this reaction had been demonstrated and it was believed that the *treponema pallidum* had been cultivated, a number of investigators — including Noguchi (73), Craig and Nichols (74), Kolmer (75), and Zinsser and his associates (47) — carried out fixations with antigens made from pure cultures of the treponemata. In our own experience, we obtained partial fixation with such organisms, but we were able to obtain similar results with antigens made of various bacteria like the colon and typhoid bacilli and convinced ourselves that here again the moderate fixation was due to the lipoidal substances in the bacterial bodies. Moreover, as we shall see below, it is quite unlikely that the culture treponema has any immunological relationship to the virulent organism of the syphilitic lesion. It is either an entirely different form or else it represents a rough form so modified by artificial cultivation that it has no immunological significance.

It cannot, therefore, be concluded from any of the evidence furnished by complement fixation reactions that a specific antibody comparable to those present in bacterial immunity develops in the course of syphilis.

The same thing applies to precipitation reactions. Fornet (76, 77, 78) and his collaborators found that syphilitic sera mixed with clear extracts of syphilitic livers similar to those used in Wassermann's early experiments led to precipitations which were not seen in similar experiments done with normal sera. Michaelis (79) observed analogous reactions. There is probably no question about the accuracy of these observations, but, again, the knowledge which we have gained concerning the nonspecific precipitation which is the first stage of a Wassermann reaction and upon which depend the numerous diagnostic flocculative tests such as those of Jacobsthal, Sachs-Georgi, Kahn, Hinton, etc. has rendered these experiments without significance for immunological processes in syphilis.

In regard to agglutination there has been much difference of opinion. Our own experience, which we believe corresponds with that of most

* See Chapter VIII for details concerning the Wassermann reaction.

investigators, was entirely negative in regard to any agglutinating action of syphilitic sera upon virulent treponema pallidum. The subject was, however, much stimulated by the cultivation of treponemata from syphilitic lesions of man and rabbits by Schereschewsky (80, 81), Noguchi (82), and by ourselves with Hopkins and McBurney (47). It is true that Kolmer (75) observed that cultivated treponemata were agglutinated in the sera of rabbits treated with culture material and similar experiments were carried on on a large scale by ourselves with the named collaborators. We could not discover any diagnostically reliable difference in the agglutination of virulent organisms, either in normal or syphilitic human or rabbit sera. Nor did we find that the avirulent treponemata were more actively agglutinated in syphilitic than in normal serum. Slight agglutination of these organisms takes place in many human sera, but with no correlation whatever to the existence of syphilis. Immunization of rabbits with culture treponemata results in a powerful antibody response to these organisms, but the antibodies so formed have no action upon the virulent treponemata and there seems to be no immunological relationship between the cultivated organism and the virulent one.

Reports have appeared at various times that virulent treponemata were immobilized in the presence of late syphilitic sera. Such results have been published by Hoffmann and Prowazek (83, 84) and were confirmed by Zabolotny (85). Negative results were obtained by Landsteiner and Mucha (86, 87). Most investigators who have repeated these experiments have failed to observe any significant differences in the action of syphilitic and normal sera upon the virulent organisms obtained from lesions and it is unlikely that such direct serum action has any practical importance in the response acquired in syphilis.

There have been a few reports stating that the sera of syphilitic men and animals exerted a treponemacidal action on virulent organisms. In 1921 Eberson (88) claimed to have demonstrated bactericidal antibodies, but those who have sought to confirm this observation nearly all failed. (See Beck (89).) Tani, Saito, and Funada (90), however, in 1935, stated that by mixing the treponema with syphilitic sera *in vitro* and then injecting the mixtures intradermally it was possible to show a destructive action of the serum on the treponema. But Beck (89), who has recently repeated these experiments using human syphilitic spinal fluid and the blood sera of both man and rabbits, has uncovered no conclusive evidence which supports the findings of the Japanese workers. The intracellular localization of treponemata in lung, liver, and kidneys in congenitally syphilitic children has been interpreted by Levaditi (91) in part as due to phagocytosis. One of us with Hopkins saw similarly situated treponemata in sections of syphilitic rabbit testes, but in studying the mechanism of the natural resistance of mice against treponema pallidum, we have

observed actively motile unphagocyted organisms surrounded by masses of leucocytes as long as three days after injection. We could not convince ourselves that any significant amount of phagocytosis had occurred. These observations are at variance with those of Hoff and Siberstein (92, 93), who found some indication that the sera of malaria-treated general paralytics contained opsonizing substance for the treponema. Beck (89), however, has reinvestigated this matter and concludes that such sera contain no opsonic elements. Occasionally he noted what appeared to be partial ingestion of an organism by a leucocyte but attributed this to an active boring movement of the spirochete into the cell.*

We believe that if phagocytosis plays a role in protection against syphilis, it is accomplished not by the mobile leucocytes but by fixed tissue elements probably of the reticulo-endothelial system.

In summary, then, it is clear that while the possibility remains that antibodies may be formed against virulent treponemata in the course of the developing resistance following infection, we have no unquestionable experimental proof of this, nor have we any knowledge of the nature of such antibodies. The possible formation of antibodies of the heterophile type through a combination of substances derived from the organisms and from the tissues of the host has been alluded to in Chapter VIII.

Attempts at Passive and Active Immunization. The failure of passive immunization with sera obtained from syphilitic men and animals is further proof that immunity in syphilis is independent of humoral factors. Many extravagant claims of success have indeed been advanced only to be refuted by accurate study. In this respect there is great similarity between the reports in the case of syphilis and those in tuberculosis and cancer. A brief examination of the bibliography in Neisser's monographs (8, 41) is sufficient to convince one of the many attempts that have been made in this direction and often by methods as ludicrous as the claims of success for which they formed the basis. The most careful and skillful workers have uniformly reported failure. Metchnikoff and Roux † treated various monkeys with blood from syphilitic patients and used the serum of these animals for protection experiments. There were a few instances in which mixtures of such serum with syphilitic virus rendered this inactive on inoculation. A powder made of this serum was supposed to have some protective effect when applied to fresh inoculation spots within the first hour after inoculation. However, injection of the serum had no effect whatever. Casagrandi and De Luca (94), using serum of a dog treated with syphilitic virus, obtained entirely

* For additional references and a more extended discussion of the possible function of the phagocytes in resistance see Chesney (26).

† See Neisser (8).

negative results, and Finger and Landsteiner (95) report negative results with monkey blood in man. The most extensive experiments were again those carried out by Neisser (8) and his associates. They were done by the treatment of animals with dead and living syphilis virus, organ extracts and the blood of syphilitic man and monkey. Horses, sheep, and monkeys were used for the production of "immune" serum. In no case was there the slightest protective effect on the part of the serum either *in vitro* or *in vivo*, and the results of the experiment were unqualifiedly negative.

The numerous efforts to actively immunize against infection have been attended by equally unsuccessful results. This fact also suggests that resistance in syphilis depends upon a different mechanism than that governing acquired immunity to the majority of bacterial and virus agents of disease.

Metchnikoff and Roux (96) were the first to attempt immunization with killed virulent organisms and filtrate material. They injected apes with material from syphilitic lesions heated at 51° C., as well as unheated filtrates from the same substances. Their results were entirely negative. Neisser (8) used extracts of primary lesions and phenolized tissues from cases of congenital syphilis. The results were inconclusive. Casagrandi and De Luca (94) treated six human beings with injections of filtrates obtained from primary lesions. Two of these people contracted syphilis in the ordinary manner. More recently a number of trials (97, 98, 99) have been made with killed treponema. All of these have failed.

In 1905 Spitzer (100, 101), following a suggestion made by Kraus, reported partially successful results in ameliorating the severity of the disease in those who had become infected in the natural manner by giving a series of subcutaneous injections of emulsions containing material from human chancres, immediately after the appearance of the primary lesion. Out of a total of 38 cases, 18 appeared to show a modification in the subsequent course of the disease, manifested by greater mildness, the absence of generalized symptoms, and in ten cases by the failure of the Wassermann test to become positive. But these results were not substantiated by others, either in animals or man. Experiments on monkeys along the same lines carried out by Neisser failed to suggest any increased resistance, nor were Brandweiner (102) or Kreiblich (103) able to confirm Spitzer's findings in man. Metchnikoff and Roux likewise failed with a similar procedure in an orang-outang.

A great many investigations have been directed toward determining whether or not cultured spirochetes can raise the resistance of animals to infection with virulent treponemata. Although, as we have remarked, antibodies are regularly produced following their injection into animals, there is no satisfactory evidence to show that the spirochetes which may

be propagated in the test tube induce any immunity whatsoever against the virulent organisms. In view of the negative results secured it would be useless to recapitulate here in detail these various reports, and the interested reader is referred to the reviews of Chesney (26, 27) and Bessemans (69, 70).

The only experiments in which *attenuated virus* has been used with some indication of success, are those of Metchnikoff and Roux (96). A laboratory assistant who had been attending to the animals noticed a small lesion on his lip which did not look like a typical syphilitic chancre. Metchnikoff and Roux carried out inoculations from the patient to monkeys and these were positive. Fournier after examining the original lesion declared it so unlike the ordinary primary sore that he did not advise treatment. No secondaries developed in the patient nor in the three chimpanzees inoculated with the material. From this occurrence Metchnikoff and Roux concluded that the patient had probably been infected in handling the monkeys, and that the virus had become attenuated by passage through these animals. On the basis of this they later inoculated a volunteer 79 years old with virus carried for five generations in monkeys. The lesion which developed was slight, consisting only of a local induration, and no generalized symptoms developed. A previous attack of syphilis Metchnikoff believes could be excluded, although, of course, this is always open to question. The experimenters suggested that the passage through lower monkeys may attentuate the virus for man.

Neisser believed that these observations should not be permitted to bear too much weight. They were made on two cases only, both of them well along in life, and the validity of the important conclusion drawn rests entirely on the always problematical fulcrum of complete exclusion of previous syphilitic infection in the two subjects. Moreover, similar attempts in this direction would involve considerable danger and it is therefore questionable whether experimentation along these lines is sufficiently promising to be justified. There is certainly no attenuation for man by passage through rabbits as has been sufficiently proven by a number of accidental infections, an instance of which is the case of a laboratory attendant reported by Graetz and Delbanco (104).

It is thus quite definite that in spite of a large amount of work by experienced observers, no reliable or even very hopeful method of active or passive immunization in syphilis has been developed.

Allergy in Syphilis. Neisser and many of the other earlier writers, it will be recalled, noted changes in the reaction capacity of the tissue cells toward reinoculation. This was frequently expressed in early infections by an accelerated pathological response, and later by the refractory state termed "anergy" by Neisser. Brown and Pearce, Manteufel and

Worms, and others have noted that resistance is much less apt to be marked following an infection which arouses little or no tissue reaction than when the response is more violent and is characterized by rather extensive lesions.* These observations all suggest that an allergic condition or hypersensitivity may be a factor in resistance, just as it has been held by so many that much of the immunity in tuberculosis is dependent upon the inflammatory response of sensitive cells. Dujardin (105) on the basis of clinical observations was the first to emphasize this hypothesis, and since then considerable investigation of the problem has been carried out, particularly by European workers. Apparently the allergy of syphilis, if it exists, is not strictly specific inasmuch as reactions may be obtained with various proteins. Bessemans and his associates (70) have studied the problem of the hypersensitivity of syphilitic rabbits to proteins, as well as to the treponemata themselves, and have reported that a definite allergic state develops in these animals. Moreover, they have asserted that local anaphylactic shock exerts a curative effect upon the syphilitic lesion (106). But Rich, Chesney, and Turner (107), in a review of the subject in 1930, concluded that allergy probably plays no important part in immunity to syphilis.

There are as yet far too little reliable data available, we believe, to warrant the assumption of any definite opinion. Even in tuberculosis, where the conditions make attempts at analysis somewhat easier, the problem of the relation of allergy to immunity, in spite of much experimentation and polemic, is still unsolved.

The Luetin Reaction. The possibility of an allergic state in syphilis early led to attempts to apply the principle of the tuberculin reaction to the diagnosis of lues. In 1909, Meirowsky (108) worked with an extract of the livers of syphilitic still-born babies. He claimed that he obtained diagnostically valuable skin reactions with this material in a considerable percentage of luetics. Similar work has been carried out by Jadassohn (11), Fontana (109), Bruck (110), and others with irregular results, so that no positive statement can be made about their value.

Noguchi (111) prepared his "luetin" with culture treponemata killed at 60° C. He used a polyvalent preparation containing all of his six strains, and since ascitic fluid was used in the cultures, he did his control skin reactions with similarly diluted ascitic fluid.

The results recorded by Noguchi and a number of others seemed at first to demonstrate the diagnostic value of such skin reactions, especially in tabes and late cerebrospinal syphilis. On the other hand, many investigators have questioned the specificity of these reactions. Moreover, it has been found by Boas and Stürup (112), by Kolmer (113), and

* See Chesney (26).

by Stokes (114) that a number of non-specific substances may produce skin reactions in luetics, indicating that the skin of such patients is more irritable than that of normal individuals. Also, Sherrick (115) has found that the administration of potassium iodide may lead to a positive luetin reaction in normal persons.

The luetin reaction is now seldom employed. Our own feeling is that there is little theoretical reason why the luetin reaction should be specific if carried out with culture organisms. We know from our own studies that, serologically and immunologically, there is no relationship between the virulent and the culture organisms. Although we ourselves cultivated a treponema indistinguishable from Noguchi's strains from the testis of a syphilitic rabbit, subsequent experimentation which showed the complete lack of relationship between non-virulent and virulent organisms has led us to question whether anyone has ever cultivated a true syphilis treponema. This, however, is an academic question. It may well be that we have all cultivated it but that, in cultivation, it has — in the language of the laboratory — gone "rough," and we know that such organisms, rough in the antigenic sense, cannot be expected to furnish specific extracts. It is still possible that a common basic protein may exist in virulent and non-virulent organisms, but it would seem to us logical to persist, in luetin experiments, with the use of virulent material obtained from syphilitic rabbits. This has been done by some workers (116) but insufficiently as yet to justify any conclusions.

The foregoing discussion manifests our almost total lack of positive information about the manner in which the body adjusts itself to the presence of the *treponema pallidum*. The single established fact is that it does make this adjustment to a greater or less degree. For the rest we have only conflicting hypotheses, which, however, being stimulants for experiment, may eventually bring order out of what is now largely chaos. The vagueness in which the subject is immersed at present is well illustrated in the concluding sentences of Chesney's Harvey Lecture: "I think of acquired immunity in syphilis as a state of resistance which evolves comparatively slowly and at its best is not always complete, general in its distribution but imparted to some tissues more than to others, probably related to the tissues rather than to the blood, but not as yet known to be the exclusive property of any particular type of cell, not necessarily dependent upon what is commonly called allergy, incapable of production by any known method of immunization, produced only as a result of disease manifestations, but not necessarily dependent upon the persistence of infection for its maintenance; finally, a state the mechanism of which is very imperfectly understood, but of surpassing interest to medical men as well as of supreme importance to the human race."

Immunity in Tuberculosis

It may be assumed with reasonable accuracy that every human being at birth is to a greater or less degree susceptible to tuberculosis, although, as we have noted in Chapter IV, it is quite likely that innate differences in the immunity of race and individual may be a factor in determining whether clinical disease will eventually manifest itself. This factor, however, is without much doubt of considerably less importance than the natural resistance acquired as a result of infection. The reasons why tuberculosis is so often arrested by the previously uninfected body when the dosage is not too great are by no means precisely defined. But it is probable that this depends upon a normal defensive mechanism primarily in the lymphatic system. Krause (117) has especially emphasized this point of view. Tuberculosis is predominantly an infection of the lymphatic tissue, he has stated, and bacilli which gain entrance through the vessels, the respiratory passage, or the intestine are carried into the lymphatics and usually held in the lymph nodes. From his extensive study of tuberculosis in the clinic and autopsy room, he believes it fair to conclude that in the great majority of infections the lymph nodes are competent to hold back the invaders and localize them. If this defense breaks down in the previously infected nodes, generalization takes place. When, however, a focus has become established and local reactions to it have taken place, an additional systemic influence is called into existence on which depends what we speak of as "immunity" or, more properly, increased resistance to further spreading of the infectious process.

Tissue Reactions in Tuberculosis. This conception agrees well with the observed facts, but it obviously fails to give us any clue to the nature of the mechanism by which the organisms, either at their first penetration of the tissues or upon reinfection, are arrested and destroyed within the lymphatic tissues. We are, nevertheless, through such careful analyses as those of Lurie which will be mentioned below, just beginning to gain some insight into the manner in which the body defends itself from the noxious presence of this parasite.

As is the case with all of basic knowledge of the tubercle bacillus and its activities, Koch (118) himself made the fundamental observations concerning immunity in tuberculosis. In 1891 he showed that if guinea pigs which had previously been infected were reinfected subcutaneously, they reacted quite differently from normal animals receiving the same doses into a similar location. This behavior, known as the Koch phenomenon, is analogous to the altered reactivity of the syphilitic animal to reinjection, with the important difference that a very marked allergic inflammatory response is a characteristic feature of the Koch phenom-

enon, which is usually absent in the case of syphilis. The details of the phenomenon are as follows. When a normal guinea pig is injected subcutaneously, little or no local reaction can be observed for 10 to 14 days, when a hard nodule appears which breaks down, leaving a persistent ulcer. The regional lymphatic glands enlarge and eventually become caseous. On the other hand, an animal which has been infected 4 to 6 weeks previously and then reinoculated subcutaneously reveals a violent inflammatory reaction at the site after the first or second day distinguished by induration, hemorrhage, and necrosis. The shallow ulcer formed by the sloughing away of the necrotic overlying tissue tends to heal quickly. Most significant is the failure of the regional glands to show evidence of involvement.

The difficulties encountered by earlier investigators who attempted to repeat Koch's experiment were most probably due to insufficient regard to virulence dosage and the time interval between first injection and reinoculation. Römer, however, soon succeeded in showing that Koch's experiment was perfectly valid and could be repeated at will provided the preliminary injection consisted either of relatively avirulent organisms or of doses of virulent bacilli which were sufficiently small to produce a long-lasting disease. The interval between inoculations, moreover, must be as long as possible — preferably six months to one year — for the best result. Löwenstein (119), who has devoted much attention to this subject, defines the difference between the responses to the first and second injections of tubercle bacilli by stating that in a properly prepared animal the inoculation gives rise to an acute inflammatory reaction instead of the chronic one characteristic of primary tuberculosis. In other words, the reinoculated animal is allergic to the material contained in the bacillus, and, as we have elsewhere remarked, the cells of the body are, as it were, on a hair-trigger, ready to respond to the stimulus of the tuberculoprotein. As far as the hypersensitive aspect of the Koch phenomenon goes, it is entirely comparable and no doubt identical with the manifestations of the tuberculin reaction.

Just what it is that determines this difference between the normal animal's response and that of the partially resistant, infected organism, is still not well understood. The allergic phenomena are so prominent in the latter that they have long been regarded by the majority of investigators as not only important but as the fundamental basis of immunity in tuberculosis. Recently the significance of the role of allergy has been brought in question, particularly by Rich and his associates. But before we discuss the problem of the relationship of allergy to immunity in tuberculosis, let us inquire concerning what is known of the actual course of events in the lesions of reinfection, for in so doing we shall learn something of the process whereby the bacilli are resisted.

Histologically reinfection has been thoroughly studied by Baldwin and Gardner (120), Krause (121), Löwenstein (119), and Long (122). Krause and Peters (123, 124) have described the differences in which a reinfection contrasts with a primary infection in respect to the immediate inflammatory reaction, the early development of a nodule, the early tendency to arrest, and the subsequent tendency to retrogression. They do not believe that this is a typical inflammatory process. In the reinoculated animal there is an early proliferation of fixed tissue cells which leads to a much more rapid formation of the epithelioid cell which, in all cases of tuberculosis, is so characteristic and which from the available evidence, as we shall see, is probably the principal destructive agent. Long (122) studied particularly the tuberculous lesion of the testis in the guinea pig. He found in reinoculated animals, within 24 hours, marked hyperemia and edema and a rapid infiltration with epithelioid and round cells. At the corresponding time in the normal animals there are hardly any changes. Thereafter in the previously infected guinea pig, coagulation-necrosis rapidly develops, followed by absorption of necrotic tissue with a continued increase in the numbers of epithelioid cells. Unless a modifying suppuration intervenes, the testis of the immunized animal within six weeks is densely infiltrated with epithelioid cells and fibrous connective tissue, with little necrosis; while that of the non-immunized animal is largely necrotic, with a much less extensive epithelioid and fibroblastic proliferation. Moreover, extension to other organs was not observed in the reinjected guinea pigs. Baldwin and Gardner's (120) observations on the events taking place in the pulmonary lesions of tuberculous guinea pigs reinfected by inhalation agree essentially with the findings of Krause and Long. A moderate localized inflammation was noted during which atypical and abortive tubercles were formed which had a tendency to remain localized. They were unable to find microscopically tubercle bacilli in these lesions, but inoculation of the material into normal guinea pigs revealed their presence, although necessarily they must have been relatively few in number.

Lurie (125) has recently laid stress on the diphasic nature of the tuberculous process. According to him and other observers, particularly if large numbers of bacilli are introduced into normal animals, the first stage is characterized by a diffuse accumulation of mononuclear cells which actively take up the organisms. This diffuse process is well illustrated by the findings in rabbits following the intravenous administration of a large dose of avian tubercle bacilli which results in the "Yersin" type of tuberculosis. There is an infiltration of the splenic pulp, liver sinuses, and the bone marrow by large epithelioid-like cells with cytoplasm engorged with innumerable organisms. The cells appear to be undamaged and caseation fails to occur. Lewandowsky

(126), whom Lurie has cited, observed a similar diffuse infiltration of the skin of guinea pigs injected intracardially with mammalian tubercle bacilli. There was here likewise an absence of necrosis. In contrast, previously inoculated animals under the same conditions exhibited a rapid formation of epithelioid nodular tubercles containing few bacilli with caseation. Here the pathological picture is one of marked localization compared with the diffuse character of the lesions in the initially normal animal. Analogous to these observations are the results of the careful experiments in rabbits of Lurie (127, 128, 129, 130), who employed the quantitative plating method for determining the number of bacilli in a definite weight of infected tissue. He has shown that both human and bovine tubercle bacilli, following their ingestion by the monocytic type of cells, multiply in them for a time without apparently injuring the cells in the slightest degree. During this interval there is a more or less diffuse accumulation of mononuclears about the focus through mitosis of the normal local cellular population. After a time, and coincidently with the appearance of hypersensitivity, mature epithelioid tubercles are formed; there is exudation of fluid into the alveolar spaces of the lung; incipient caseation, and, most significantly, the destruction of organisms, as indicated by microscopic examination and the results of cultivation. Here again the two stages of the infection are clearly demarcated. Thomas (131) has likewise described the same evolutionary stages in hematogenous experimental tuberculosis of the rabbit. Lurie believes that the monocytic type of cell under the influence of certain constituents of the bacillus liberated after death [possibly the phosphatide fraction; see Smithburn and Sabin (132)] is converted into the epithelioid cell. However this may be, it is clear from Lurie's work that following a brief period of multiplication the organisms for the greater part are then destroyed. From the fact that the bacilli at this time are situated within the characteristic epithelioid cells of the tubercle, it is also fairly certain that these cells represent an essential factor in the mechanism of destruction. In the animal rendered resistant by a previous infection, the assemblage of monocytes, their transformation to epithelioid cells, the formation of typical tubercles, and the decrease in the number of bacilli all take place much more rapidly than in the normal animal infected for the first time. The fibroblastic proliferation is also accelerated and there is the swift mobilization of polymorphonuclear leucocytes common to the acute inflammatory reaction irrespective of its origin. The acceleration in the sequence of all these morphological changes has, of course, been emphasized by other workers (133, 134, 135) in addition to those already mentioned.

These histological analyses have revealed two processes by which the tubercle bacilli are opposed by the host. The first, long recognized by

many authors, consists in the segregation and walling off by means of the anatomical structure of the tubercle. This is in essence simply a mechanical effect brought about by the barrier of epithelioid and fibroblasts. It represents a specialized case of the general phenomenon of inflammatory fixation which has been especially emphasized by Krause (121), Opie, and by Menkin.* The second consists in the phagocytosis by macrophages which at first apparently lack the capacity to kill the bacilli which they have ingested but which later, due to the stimulus emanating from the latter and which must be regarded as a process of acquired immunity, become altered in such a manner that they are able to destroy considerable numbers of the parasites.

Two questions immediately arise in connection with these factors of resistance. To what extent if any do humoral elements play a part, and what is the significance of the allergic state in this immune response?

Antibodies and Immunity in Tuberculosis. That certain antibodies are formed in tuberculosis as well as following the injection of killed organisms into normal animals there can be no doubt. The tubercle bacillus in its antigenic properties is similar to other bacteria, containing at least three principal kinds of antigens — proteins, carbohydrates, and lipoidal elements which act like haptenes. (See Chapter II.) Agglutination, precipitation, complement fixation, and opsonic properties can be detected in the sera of animals injected with killed tubercle bacilli. In the sera of tuberculous individuals certain of these effects can also be frequently demonstrated, particularly those of complement fixation † and opsonification. Also antibodies capable of passively inducing anaphylaxis in guinea pigs have been regularly demonstrated in the sera of vaccinated animals and occasionally in those of infected guinea pigs by Enders (136), Branch (137), Lewis and Seibert (138), and others. But whether or not any of these various antibodies play a role in resistance is problematical. Neither lysins nor bacteriostatic principles active against the tubercle bacillus have been demonstrated *in vitro*, nor have the results of many protection and therapeutic tests with immune sera indicated any possibility that the antibodies which they contain could passively confer an increased resistance. Moreover, the serum antibodies have no capacity to induce passively the tuberculin type of hypersensitivity which may be of importance in immunity. Although the opsonins can increase the phagocytosis of tubercle bacilli both by polymorphonuclear leucocytes and macrophages,‡ we are not aware of any evidence which indicates that the former type of cell can kill the bacillus under any circumstances whatso-

* See references 145, 146.
† For literature see Calmette (135).
‡ See Chapter XI.

ever nor that the presence of opsonic antibody promotes its destruction following ingestion by the monocytic or epithelioid variety of cell.

It is thus generally accepted that the known antibodies which react either with the bacilli or products derived from them, unlike those against many other bacteria, are unable to enhance the resistance of the animal. But some recent observations of Lurie (139, 140) suggest that certain unknown elements appear in the body fluids of infected animals, which seem to have an inhibitory effect on the growth of the tubercle bacillus. He mixed the organisms with melted agar and injected the mixture subcutaneously in normal and "immune" rabbits. The cells penetrate the agar mass slowly, whereas the fluids enter readily. By removing the agar at intervals and determining the number of organisms and microscopic colonies which had developed, Lurie found that growth had proceeded unchecked in the normal animal, whereas in the "immune" there was a very marked decrease in the bacterial population of the agar. These results were further substantiated by experiments in which collodion-impregnated silk bags containing agar and bacilli were inserted into the peritoneal cavities of rabbits. By this means all cellular invasion was eliminated, but the same inhibitory effect was observed in the bags which had been kept in the bodies of "immune" rabbits. The findings are most significant provided they can be repeated using a large number of animals of various species. In this connection should be mentioned the experiment of McJunkin (141), who noted granulation and other evidence of degenerative changes in the bacilli injected in large quantities into the peritoneal cavities of tuberculous guinea pigs. Furthermore, he asserted that with the filtrates of the peritoneal fluid he could induce the allergic state in normal guinea pigs, which indicates that it contained constituents of the bacterial bodies and thus suggests that dissolution of the organisms takes place under these conditions. However, in the McJunkin experiment it is impossible to eliminate the possibility of participation by the phagocytes.

Thus, in spite of the fact that we must at the moment adhere to the traditional conception of the inadequacy of antibody reactions in immunity to tuberculosis, the possibility should be nevertheless considered that the body fluids of the immune animal may in some cases contain factors which are unfavorable to the unlimited proliferation of the tubercle bacillus.

Allergy and Immunity in Tuberculosis. Opinion is sharply divided among students of tuberculosis as to whether the allergic reaction is a factor in the increased resistance to reinfection or whether it is a concomitant but actually independent phenomenon. The problem has always been of interest but of late years has been accentuated by Rich's *

* The most recent statement of Rich's position, together with numerous references to the literature, may be found in a paper in the Revue d'Immunologie (1937) (142).

insistence upon the validity of the latter alternative. The earlier work of Krause and Willis (143, 144) had shown that the acute inflammation of allergic tissues resulted in the localization of the bacteria at the site of inoculation for many days before they spread to the regional lymphatic glands in contrast to the swift emigration of the bacilli in normal animals. Opie's (145) experiments on the Arthus phenomenon showed that the protein to which the animal was sensitive was fixed *in situ* by the local inflammation of the Arthus phenomenon. Menkin (146) has likewise demonstrated the fixation of a variety of materials including horse serum dyes and various bacteria in inflammatory areas caused by different kinds of injurious agents such as turpentine and other simple irritants as well as pyogenic bacteria like staphylococci. Freund (147) has found that tubercle bacilli of moderate virulence (human strain) are held in the skin of rabbits repeatedly vaccinated with killed organisms, although it must be admitted little or no localization was observed with the more virulent bovine strain. The Koch phenomenon, itself so manifestly allergic in its obvious characteristics, has been of course a potent factor in guiding the thinking of the investigators who have held the view that immunity in tuberculosis is practically synonymous with allergy.

The question has remained so long unanswered because of the experimental difficulties in producing allergy without immunity or *vice versa*. The conditions which inaugurate the one (*i.e.*, the formation of the tubercle) are apparently essential for the development of the other. Allergy and immunity go hand in hand in most cases, although in naturally resistant animals such as the rat and the mouse it is to be noted that allergy does not readily develop. Of late, however, there have been a number of attempts to desensitize allergic animals (guinea pigs and rabbits) by frequently repeated and increasing doses of tuberculin. Thus Rothschild, Friedenwald, and Bernstein (148) have been able by this procedure to obtain apparently complete desensitization of the skin to tuberculin. Upon testing the immunity of such animals by reinoculation they found that the elimination of the allergic response, although it profoundly influenced the reaction in the direction of greater mildness and far less destruction of tissues, did not deprive the treated animals of any aspect of their immunity as measured by the course and severity of local lesions, by the spread to the viscera, and by the severity and progress of the lesions set off there compared with untreated tuberculous animals. Similar findings have been reported by Cummings and Delahant (149) and Derrick, Branch and Crane (150). These results have been criticized by Topley and Wilson (151) and by Opie (152) on the ground that the repeated injections of tuberculin may have increased the resistance of the animals in addition to bringing about desensitization.

Even if this were so it does not appear to us to invalidate the conclusion that a considerable degree of immunity persists in the apparent absence of sensitivity to tuberculin. Of greater weight are the observations of Woodruff and Willis (153, 154, 155), who instead of killing their animals after a time as did Rothschild and his collaborators, in most cases allowed them to die from the infection. They then found that the desensitized animals lived a shorter time, exhibited more massive involvement of the lung, and that their organs contained many more tubercle bacilli than the allergic controls. In subsequent experiments they carried out histological studies of the organs of desensitized and allergic guinea pigs and noted that the pulmonary disease of the former group was far more extensive and that the lesions contained an overwhelming accumulation of acid-fast bacilli. However, the liver and spleen showed no definite formation of tubercles. In the allergic animals these organs were involved but their pulmonary disease was much less.

On the other hand, Branch and Enders (156) have shown that following intravenous or intramuscular inoculation with killed tubercle bacilli, whereas a considerable degree of resistance as measured by the survival time following subsequent infection may develop, many of the animals fail to become allergic. These experiments imply that a preliminary state of sensitivity is no requisite for the development of immunity, although before we can be certain, more experimentation of the same sort, using greater numbers of animals, must be carried out. The experimental attempts, then, that have thus far been made with the object of resolving this problem have yielded conflicting results. But to our minds the weight of evidence inclines somewhat to the side of those who contend that allergy is not an important factor in resistance. Certainly in the case of other diseases, particularly in those in which immunity may be passively transferred as in pneumococcus or streptococcus infections, it is quite clear, as Rich (157, 158) and others (159, 160) have pointed out, that although hypersensitivity may develop, it is not at all essential to the presence of a very solid resistance. Very possibly if we could produce an immunity of that degree against infection with the tubercle bacillus a similar independence of the two phenomena might be more readily demonstrated.

Although there has been a considerable amount of clinical and epidemiological data collected with the object of settling this question in respect to tuberculosis in man, they are perhaps even more contradictory and confusing. The method employed in all of these studies has been to determine whether or not the tuberculin test was positive or negative in a group of persons about to be exposed to more or less the same environmental conditions and then to observe during subsequent periods the numbers in each group which developed clinically apparent disease.

In certain cases, such as some of those of Heimbeck, vaccination with B.C.G. was carried out in the non-reactors — a number of whom still failed to become tuberculin positive.

Heimbeck (161, 162, 163) in Norway during the six years from 1927 to 1933 studied in this manner 762 probationer nurses who entered the hospital at Oslo. Of these, 266 were negative reactors. By 1933, 118 of the total number had developed signs of tuberculosis, of which 94 belonged to the negative group upon entrance. Among a group of 94 persistent negative reactors immunized with B.C.G. 22 developed tuberculosis, while out of 164 who gave positive tests following vaccination only 3 became ill. It should be emphasized, however, in attempting to evaluate the significance of these figures, that Heimbeck has included as tuberculosis a large number of cases of erythema nodosum, which may not be due to infection with the tubercle bacillus, although Heimbeck so believes. Topley and Wilson (151), who have summarized much of the literature, also cited Scheel (164), who has reported similar findings in a group of medical students at Oslo. Jacobson (165) in Denmark, however, was unable to find any definite correlation between the result of tuberculin testing and the following incidence of manifest disease in 147 probationer nurses, while Shipman and Davis (166) in California reported that a larger percentage of those exhibiting a positive reaction developed tuberculosis. The same tendency appears to be shown by the negro workers in the Witwatersrand Mine in South Africa (167). In support of Heimbeck's conclusions, however, is the recent careful investigation of Plahiff (168) in the Mental Hospital in Kingston, Jamaica. Among a group negative to 1.0 mg. of tuberculin upon entrance and which spent a total of 202.5 person years in the institution the mortality from tuberculosis was 9.9 per cent per annum. Contrasting with this group was that comprised of those positive to 0.01 mg. of tuberculin who spent a total of 1415.24 person years in the hospital with an annual mortality rate of 1.9 per cent. Intermediate was the group positive to 1.0 mg. but negative to 0.01 mg. of tuberculin which spent 505.5 person years in the institution with an annual mortality of 4.5 per cent. Finally we should mention the very extensive work carried out by Meyers and his co-workers at the Lymanhurst School in Minneapolis. From his analysis of the tuberculin tests and X-ray findings, together with their subsequent histories, in the cases of many thousands of children Meyers is very definitely of the opinion that early infection with its resulting sensitivity offers a more favorable basis for the future development of active disease. We lack space here to present his findings in any detail and refer the reader to his recent monograph (169).

We are thus once again confronted with evidence on both sides which renders a final decision impossible. On the whole it would seem that a

primary infection successfully overcome increases the resistance of an individual. This view is supported by the enormous data obtained at autopsy and by the well-substantiated dictum of Marfan concerning the resistance of those who present evidence of healed glandular tuberculosis. Moreover, this attitude is harmonious with the data from animal experimentation. But in the last analysis such epidemiological studies cannot, it seems to us, settle the fundamental point at issue. A positive tuberculin test indicates the presence of allergic sensitivity, it is true, but it also indicates that infection has occurred from which may arise wholly independently an exaltation in the resistance of the host. Since here allergy and immunity coexist and we have no means of separating them, it is rash to draw the conclusion that they are causally related or, on the other hand, that they are actually antagonistic as Rich and Meyers assert.

As always, the truth is probably to be found midway between these extremes. Provided the allergic response is not too violent and destructive to tissue, it results in the formation of a barrier, as Krause and the others have maintained, to the dissemination of the tubercle bacillus. But if it results in excessive injury, it may do more harm than good. Thus Lurie (125) has stated that when the organisms are relatively numerous and the resulting reaction extremely pronounced, the increased lymph flow resulting from the intensified inflammation may sweep the bacilli to the draining lymph nodes with even greater rapidity than in the normal animal.

If we regard the result of the hypersensitive response as consisting only in a "fixation" of the organisms, it seems to us that this in itself, although of temporary value, would be insufficient to yield any adequate form of immunity. Indeed we feel that the experimental proof of the capacity of the body to destroy the bacilli as well as merely to restrict their dissemination is enough to force us to conclude that allergy cannot be the only factor in resistance and probably is not even that which is essential.

The ultimate solution of the problem is greatly to be desired, not only because of theoretical considerations but because it will furnish a better conception of how to deal directly with the disease. For it is apparent that if allergy is found to play only a very minor role or no part at all in resistance, it would be well to try to determine how an individual might be safely and conveniently desensitized, since as Rich (142) has remarked, all would recognize that the reactions of hypersensitivity in themselves are undesirable and injurious.

Attempts to Immunize against Tuberculosis. Efforts to actively immunize against tuberculosis have been so numerous that it is quite impossible to do justice to the large literature in a short summary. For details and a more complete discussion we may refer to Löwenstein's

treatise (119), the papers of Krause (170), Long's (171) article of 1926, a review of Petroff (172), and Calmette's book (135).

Three general methods have been employed which included the administration of killed organisms, living attenuated bacilli, and living virulent bacteria in minute doses.

Vaccination with Killed Bacteria. Koch early attempted to immunize with organisms killed by boiling but soon abandoned the method as unpromising. This line of investigation was, however, continued by Löwenstein (119), by Calmette, Guerin, and Breton (173), by Uhlenhuth (174), and by Uhlenhuth, Müller, and Grethmann (175) with inconclusive results. Trudeau (176) found an increased resistance following the injection of heat-killed cultures, but believed it to be less than that obtained in animals by treatment with attenuated living organisms. Experiments by Calmette and Guerin (177) on cattle and of Loeffler (178) on dogs were also on the whole encouraging, although Calmette finally came to the conclusion that killed vaccines were useless.

Zinsser with Ward and Jennings (179) and Petroff and Stewart (180) showed that guinea pigs vaccinated with killed cultures would outlive normal control animals by a considerable period of time. Since that time these findings have been confirmed by a number of observers. Petroff, Branch, and Jennings (181) extended Petroff's original investigations and obtained similar results in a large number of guinea pigs. Soper and his associates (182) carried out a comparative study of the effect of immunizing rabbits with virulent killed bovine bacilli, attenuated bovine organisms (B.C.G.), and a virulent living human strain. The virulent killed bacteria appeared to be quite as effective in raising the resistance as the living attenuated organisms, but neither of them was so efficient as the human strain. Branch and Enders (156) employed bacilli killed by heating at 65° C. for one hour and by treatment with 0.3 per cent formalin. The heat-killed vaccine was administered by three routes: the intravenous, the intramuscular, and the intraperitoneal. A marked degree of dermal allergy resulted only in the group of animals injected intraperitoneally, but all three groups outlived the controls by a significant number of days (the survival time was roughly twice as long). A similar increase in resistance was observed among the animals treated with the formolinized vaccine. These observations, although suggestive, must be interpreted with caution because of the small number of animals employed.

An extensive investigation on the immunizing capacity of heat-killed organisms has been recently reported by Opie and Freund (183) with the rabbit as the experimental animal. Their results indicate with great clarity that a vaccine of this kind is effective, particularly when administered together with a foreign protein like horse serum.

Only a few trials of killed vaccines have been carried out in human beings. Maragliano (184) in 1903 introduced a glycerinated emulsion of tubercle bacilli killed by heat into the scarified skin of the arms. No statistical comparison of the results was made, nor was the value of the method tested in animals, but Borsarelli (185) vaccinated 95 newborn infants in this manner and followed their subsequent histories for two years. He found no difference between the morbidity and mortality in this group compared with non-vaccinated children. Langer (186) has likewise attempted the vaccination of infants with killed bacteria, but the numbers involved are too few to warrant any definite opinion concerning the value of the procedure.

Within the last few years Plahiff (168) has carried out a carefully controlled study on a considerable population exposed to unusually severe risk of infection in the Mental Hospital at Kingston, Jamaica. Tuberculin tests were done on all those admitted to the institution, and the negative reactors were divided into two groups. One of these was then vaccinated with heat-killed tubercle bacilli. The vaccination was repeated until allergy developed as indicated by a positive skin reaction to tuberculin. The other group was not vaccinated and served as a control. During the period of observation, which for some but by no means all of the individuals in both groups extended over two years, the annual mortality rate from tuberculosis among the non-vaccinated was 9.9 per cent and among the vaccinated 3.7 per cent. The most striking difference between the groups was noted at about the end of one year, when the annual mortality rate of the non-vaccinated who had spent this time in the hospital was 24 per cent compared with 4 per cent among the vaccinated group who had remained in the institution during the same period. The total number of person years spent by the non-vaccinated group in the hospital was 202.5 and for the vaccinated 240.25, and the total number of individuals in each was 497 and 578 respectively. These results seem to be most important and indicate clearly the necessity for further researches of the same kind carried out on a larger scale.

Vaccination with Attenuated Living Bacteria. Attenuated cultures have in the past frequently been used as vaccines, especially in the attempt to protect cattle against infection. Such work was begun as early as 1891 by Grancher and Ledoux-Lebard (187). It was carried on extensively by Trudeau (188, 189) and since then by von Behring (190), Neufeld (191, 192, 193), Klimmer (194), and many others. Methods of attenuation consisted either in bringing about a loss of virulence by repeated subculture on artificial media carried out for prolonged periods of time, by cultivation on unfavorable media, and by treatment with various chemicals such as the halogens. Rabinovitch (195), for example,

succeeded in attenuating the virulence by cultivation in the presence of low concentrations of formalin. Frequently the human type of tubercle bacillus the initial virulence of which is low for cattle was still further attenuated by some such procedure and then used to vaccinate these animals. This was the principle underlying the preparation of von Behring's "bovo-vaccine." Similarly MacFadyean (196) proposed the use of avian bacilli as a vaccine for cattle.

The most extensive investigation on immunization with attenuated cultures is that of Calmette (135, 197), who applied the method to the vaccination of human beings. The vaccine is prepared from a culture of a strain of bovine tubercle bacilli which was isolated in 1908 and has been carried since then on a medium consisting of glycerin, bile, and potato. At the present time it appears to have largely lost its capacity to produce progressive disease in all animals, including the susceptible guinea pig. The vaccine which is known as B.C.G. (bacille Calmette-Guerin) has been administered *per os* to a very large number of infants in France and elsewhere following the earlier animal experiments of Calmette, who reported that after the administration of 50 mg. of the vaccine subcutaneously calves became resistant to a dose of virulent bovine bacilli which would kill normal animals within two months. The literature on this subject is now extremely large and it cannot be reviewed here in any detail. Most of it deals with two important aspects of the method. Is the resistance significantly increased following vaccination with B.C.G. and is it capable under any circumstances of reverting to a virulent state and causing progressive disease? The literature up to 1934 dealing with these points has been well reviewed by Irvine (198). At that time he concluded that the virulence of B.C.G. was not permanently fixed but that under suitable conditions of cultivation it might increase. Furthermore it might rarely produce progressive tuberculosis in animals but it had not been proved to have caused tuberculosis in man. As far as its value as an immunizing agent is concerned, Irvine considered that it produced in cattle a definite degree of immunity, while in other animals the results were still equivocal. In man it seemed to have increased the resistance but not so clearly that it was possible to warrant making fundamental changes in the accepted methods of dealing with tuberculosis. Shaffer (199) has also analyzed the experiments of others on the reversion of virulence in B.C.G. but in his own work was unable to obtain any indication that this could be effected.

Park and Kereszturi (200) in this country have been carrying out an experimental study of the vaccine under carefully controlled conditions during the last eight years, during which they have administered B.C.G. to 1445 infants. The earlier vaccinations were administered by the oral route according to the recommendation of Calmette, but later they

were given either intracutaneously or subcutaneously. On the basis of their experience they have stated that they consider the B.C.G. vaccine harmless both for animals and for human beings. Of particular interest is their observation that B.C.G. recovered from cold abscesses in man caused by vaccination could not be shown to have increased at all in virulence by residence in the body for periods of one to ten months. Their opinion concerning the innocuity of the vaccine is now widely shared by most all observers. For a time the disaster at Lübeck (201), where 72 out of 251 vaccinated infants died of tuberculosis, shook the faith of many, but this has been shown to be almost certainly due to contamination of the vaccine with a virulent organism. In addition to convincing themselves that the B.C.G. vaccine was safe, Park and Kereszturi believe that their data which they have submitted to statistical analysis indicate that the resistance to tuberculosis is considerably increased and they urge its use as a public health measure in the prevention of the disease in those who not yet infected may later be exposed to tuberculosis through contact with tubercular members of the same family. Among 690 vaccinated infants 6 deaths from tuberculosis occurred, contrasting with 20 deaths among 755 non-vaccinated control cases. The percentage difference in the mortality of the groups is 1.8 per cent $\pm$ 0.47, or 3.9 times the probable error. These differences, then, are most probably significant, although according to Bogen, who carried out the statistical analysis, they are not conclusive. Obviously it would be more convincing if there had been more deaths from tuberculosis among the controls. Similar results have been reported by Aronson and Dannenberg (202), although the numbers both of vaccinated and controls were far less. The huge amount of material purporting to support the value of B.C.G. vaccine published in France has been analyzed by a number of statisticians (203, 204, 205), who have concluded that it is of little value as evidence mainly because of the unreliability of the figures for the mortality rates of unvaccinated infants reared in tuberculous families which have been accepted by the French writers as the data representing the control groups. For references to the French literature the reader may consult Calmette's own summary.

We believe, on the whole, that the evidence at present shows B.C.G. vaccine to be harmless when administered to human beings, although it must be remembered that troublesome small abscesses may persist for months at the site of inoculation if the vaccine is administered parenterally. In spite of its apparent innocuity, we are of the opinion that it would be advisable to abandon its use on the ground that there is still a chance, however remote, of its becoming more virulent, provided an equally efficient immunizing agent in the form of a killed vaccine should

become available. The promising results of the recent animal experimentation with materials of this sort and Plahiff's findings in human beings, to which we have alluded, hold out the hope that we may indeed have such a vaccine in the form of heat-killed suspensions of virulent organisms.

In regard to the value of B.C.G. as an immunizing agent, we may conclude that it definitely increases the resistance to a moderate degree, but that it has not been shown in this respect to be decisively superior to killed vaccines.

Vaccination with Living Virulent Organisms. This method was proposed by Webb and Williams (206, 207), who by means of Barber's technique of single-cell isolation injected guinea pigs with an initial dose of only one or two organisms and then gradually increased the numbers until as much as 141,000 bacilli were introduced without producing progressive disease. The experiments were repeated in rabbits by Lieb (208) with analogous results. Webb and Williams (208a) also vaccinated two children, beginning with a single organism and increasing the dose until 150 bacteria were administered. No tuberculosis resulted from this dangerous procedure, but since the subjects failed to develop positive tuberculin tests, it seems doubtful that it could have been effective. In a third attempt made by Webb and Gilbert (209) local lesions with extension to the lymph nodes occurred, and the tuberculin tests became positive. Save for this the child apparently remained well.

More recently Selter (210, 211) attempted the immunization of nine infants by this method. The tuberculin reaction became positive, and localized lesions developed and no progressive tuberculosis supervened.

It is hardly necessary to remark that this method is far too dangerous, even if it were proved to be effective, to warrant for a moment consideration of its use. The experiments are of some interest, however, as they afford direct evidence of the high natural immunity of human beings to relatively small numbers of this organism.

Attempts at Passive Immunization and Serum Therapy. Passive immunization has met with no success. As we have seen, antisera which react in various ways with the tubercle bacillus or its products may be produced in animals, but they have never been shown to have exerted any curative action. Maragliano's and Marmorek's sera, and a number of others which had a considerable vogue for a time, have been found quite useless.*

* For a full account of the various methods of preparing the antisera which have been employed in attempts at passive therapy see Calmette (135).

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CHAPTER XIX

DIPHTHERIA

DIPHTHERIA is a unique disease from an immunologic point of view in that much of our important and practical knowledge of it was accumulated very early in the development of the science of bacteriology and immunology. Being an almost pure toxemia, the disease lent itself to a rapid development of knowledge during these pioneering researches. Furthermore, there is perhaps no other infectious disease which has had and is receiving so much investigative study as diphtheria.

The gradual reduction of the amount of diphtheria throughout the world is, in large part, due to the important preventive measures that have been developed against it. Table I illustrates the striking reduction in the amount of diphtheria which has occurred in the United States during the past forty years. Similar reductions have been experienced in other countries. It cannot be said, however, that this reduction can be attributed to the completeness with which large populations are artificially actively immunized. There are undoubtedly many obscure biological factors which play a role. The sudden appearance in recent years of many cases of so-called malignant diphtheria in many European districts is a case in point. It should be emphasized that death rates only are given in Table I. Since there is a mortality rate of about 6 per cent even in adequately treated cases, these figures can be multiplied by 15 to get an approximate morbidity rate. If this is done, it can be seen that diphtheria is still far from being extinct.

The Action of Diphtheria Toxin. In diphtheria practically all the clinical symptoms (except the mechanical ones which result from inflammatory ulceration of the mucous membranes of the throat and pharynx or occlusion of the air passages by similar membranous inflammation) are the result of the absorption of the specific diphtheria toxin.

Diphtheria toxin is a potent tissue poison. When a suitable dose is injected subcutaneously in a guinea pig, there is first a delay of several hours before changes are grossly apparent. There is then a considerable accumulation of gelatinous edema with some hemorrhage. If a large dose of toxin has been administered, the animal may die before the edema becomes extensive. With a small dose of toxin, however, this edema is followed by a marked inflammatory reaction with tissue necrosis and gangrenous sloughing.

TABLE I

DEATH RATES FROM DIPHTHERIA (INCLUDING CROUP) PER HUNDRED THOUSAND POPULATION IN SOME LARGE CITIES OF THE UNITED STATES

CITY	1935	1934	1930–1934	1925–1929	1920–1924	1915–1919	1910–1914	1905–1909	1900–1904	1895–1899	1890–1894
Rochester . . .	0.0	0.3	0.7	7.5	16.9	12.7	22.1	32.4	32.3	45.9	96.6
Syracuse . . .	0.0	0.0	0.4	2.0	22.9	12.9	16.6	17.4	17.7	31.1	55.4
New York . . .	0.9	1.4	2.2	10.7	14.0	21.8	28.0	40.0	58.0	85.8	134.4
Philadelphia . .	1.0	1.1	1.3	11.8	16.7	22.7	24.6	34.1	50.0	100.6	119.4
Boston	1.3	1.1	3.2	8.3	20.2	26.3	20.0	26.2	53.7	83.9	112.2
Detroit	0.7	0.7	4.8	19.7	24.3	32.2	33.3	22.6	38.5	62.9	132.9
Milwaukee . . .	0.7	1.5	2.0	8.5	11.4	19.8	27.8	26.4	22.7	51.7	116.2
Chicago	2.4	1.2	4.3	11.7	17.5	31.2	37.9	27.0	33.9	69.7	117.3
Omaha	3.2	2.7	4.7	6.4	22.9	35.8	15.8	24.5	20.5	28.2	82.9
Denver	4.8	4.4	3.9	8.9	23.2	6.7	10.2	20.8	29.6	27.3	130.2
San Francisco . .	0.6	0.1	1.2	4.6	23.0	17.0	9.2	14.4	44.2	21.6	54.8
Los Angeles . .	2.8	3.0	4.8	7.0	14.4	7.1	7.5	15.3	25.4	35.8	46.0
Knoxville . . .	13.6*	10.8	9.6	6.3	11.2	—	—	—	—	—	—
Atlanta	6.8*	7.5	5.7	7.0	13.3	10.1	12.5	14.2	11.1	10.5	8.8
Birmingham . .	2.9	2.9	4.2	5.4	5.3	7.2	8.3	6.2	13.4	16.5	20.6
San Antonio . .	9.4	2.0	5.6	10.3	7.7	8.6	6.7	7.6	17.1	20.6	4.4
Dallas	6.5	6.5	9.7	9.8	8.3	7.4	6.9	8.1	16.9	16.0	21.8

* One third or more of the reported diphtheria deaths were stated to be in non-residents.

Compiled from American Medical Association Report (1).

The most characteristic finding in guinea pigs is met with in the adrenal glands. Extravasations of blood are found in the cortex and medulla, being more marked in the latter, although the actual histological changes may be more marked in the cortex.

Severe changes may occur in the heart in man. At first the changes are parenchymatous, with edema of the muscle fibers and congestion of the vessels. This is followed by marked acute inflammatory infiltration resulting in the so-called diphtheritic myocarditis which is such a serious problem in the management of clinical cases. A characteristic hemorrhage is found in the apex of the heart of the guinea pig when death occurs on the second or third day.

Diphtheria toxin has a specific affinity for the nervous system of man. Some degree of paralysis occurs in a considerable percentage of cases. The paralysis affects, in the order of frequency, the palate, muscles of accommodation, limbs, trunk and neck, larynx, and external ocular muscles. The lesion is typically a peripheral neuritis. Of considerable interest is the lateness in the course of the disease at which paralyses occur. The first involvement is usually of the palate or ciliary muscles toward the end of the third week. Other paralyses may appear from

this time until the eighth or ninth week. The reason for this delayed appearance is not clear. It may take considerable time for a sufficient amount of toxin to accumulate in the nerve tissue before its effect becomes evident. On the other hand, administration of antitoxin late in the disease but before any evidence of neurological symptoms will not prevent their appearance.

Many cases of post-diphtheritic paralysis occur in patients in whom the actual infection, occurring a few weeks previously, was very mild. Very often such infections are so mild as to be unnoticed. An analogous situation exists in the production of experimental paralysis in the animal. To be successful, partly neutralized toxin should be used. In a study of experimental paralysis Ramon, Debré, and Uhry (2) used a toxin that was partly detoxified by formalin. They found that active immunity may develop while the paralysis is incubating. In fact, the blood of some animals contained considerable amounts of antitoxin while the paralysis was extending. Such experiments demonstrate the futility of the late administration of antitoxin as a therapeutic procedure at the time of appearance of post-diphtheritic paralysis.

Another characteristic of diphtheria toxin which has an important bearing on the management of patients is the rapidity with which toxin is fixed by the tissues. Once fixed, it is practically impossible to neutralize it with even enormous doses of antitoxin. Many experiments (3, 4, 5) illustrate this fact. In one of these a rabbit which had received ten fatal doses of toxin intravenously was given a total of 500 antitoxin units in divided doses as follows: 100 units after 20 minutes, 100 after 40 minutes, and 150 units each after 60 and 80 minutes. This rabbit died. Another animal given the same dose of toxin received 200 units of antitoxin 20 minutes later and lived. The amount necessary to save life in rabbits receiving 10 fatal doses intravenously was as follows:

Given after 10 minutes — 5 units antitoxin
Given after 20 minutes — 200 units antitoxin
Given after 30 minutes — 2000 units antitoxin
Given after 45 minutes — 4000 units antitoxin
Given after 60 minutes — 5000 units antitoxin
Given after 90 minutes — No amount

Glenny and Hopkins (6) investigated the effect of antitoxin injections upon the lesions produced by intradermal injections of a Schick dose of toxin. They showed that if a Schick dose of toxin is given intradermally, a small reaction will appear, even if ten thousand times its equivalent of antitoxin is injected intravenously fifteen minutes later, or a million times its equivalent in antitoxin is given in the same way thirty minutes later. The size of the reaction produced is diminished if 1000 units of antitoxin are injected intravenously within two and one-half hours or

intramuscularly within one and three-quarters hours, but the subcutaneous injection of antitoxin fifteen minutes after the injection fails in any way to modify the reaction.

In experiments of this type the distinction between actual fixation of toxin by tissues and early inapparent damage to tissues should be borne in mind. It is likely that such tissues are quickly damaged even though no pathologic evidence for it exists at the moment. Antitoxin cannot be expected to repair actual tissue damage.

There is a definite incubation time before diphtheria toxin will kill animals even when administered in large doses. Glenny (7) found that the minimal lethal time for guinea pigs was ten hours. The lethal time depends upon dosage and, to some extent, weight of the animal, though this is of secondary importance, and follows roughly the formula $\log d\,(T - 10) = 12$, when d is the number of theoretical minimal doses and T is the lethal time in hours.

There is very little evidence that the diphtheria bacillus has any invasive properties. There have, however, been a few reports of bacteremia due to this organism. Chi-Wu and Ts'un (8) reported a fatal case of bacteremia due to the "gravis" type of *C. diphtheriae.* Ciantini (9) reviewed the literature on this question and reported isolating the organism by blood culture from 3 cases out of a series of 58. An interesting case has been described by Chiari (10) of a ten-year-old girl with faucial diphtheria who died on the fifth day of the disease. A membrane-like formation was found covering the mitral valves, in sections of which diphtheria bacilli were recognized in large numbers. Such isolated observations as these are extremely rare and do not invalidate the general rule that all the important damage (except mechanical) in diphtheria is due solely to the action of the toxin. Krolmer (11) published a report of a careful bacteriological and histological study of the organs of ten fatal cases of diphtheria. The organism was demonstrated in one or more of the organs in every case. On the other hand, histological examination of these organs revealed no evidence that the deposited organisms caused any damage. He felt that the presence of the bacilli in the organs was of no pathologic significance.

There is evidence, in some animals, of a local immunity of the mucous membranes of the upper respiratory passages against the direct implantation of diphtheria bacilli even in the complete absence of circulating antitoxin. Traumatization of such tissues is necessary before the organism will implant itself. Although the guinea pig is extremely susceptible to the injection of a small amount of diphtheria toxin, Dold and Weigman (12) were unable to produce either clinical diphtheria or death in this animal by the massive inoculation of the nose and throat with virulent organisms. They were unable to infect monkeys in a similar experiment.

On the other hand, Guthrie, Marshall, and Moss (13) produced typical diphtheria in healthy susceptible human volunteers by inoculating their throats with virulent diphtheria bacilli.

Natural Antitoxin to Diphtheria. Diphtheria is primarily a disease of early childhood. As age advances the disease is less frequent. Furthermore, infants under 6 months of age seldom contract the disease. The reasons for this age-incidence of the disease became clear with the development of methods for titrating antitoxin and with the discovery of the Schick test.

The placental transmission of diphtheria antitoxin has been demonstrated by Fischl and Wunscheim (14) and Polano (15). Von Groer and Kassowitz (16, 17) and Kuttner and Ratner (18) showed that the amount of antitoxin in maternal blood and in umbilical cord blood is practically identical. Neill, Gaspari, Richardson, and Sugg (19) made measurements over an 18-months period of time on a mother and infant, the mother possessing an unusually high degree of immunity. They demonstrated antibacterial as well as antitoxic antibodies in both mother and infant.

Many studies have been made of the transplacental transfer of antitoxin in actively immunized animals. Richardson (20) showed that both antibacterial and antitoxic antibodies were found in the offspring of actively immunized guinea pigs. Ogawa (21) reported studies in actively immunized rabbits. The amount of antitoxin in the sera of the offspring was proportional to that in the mother. The antitoxin did not pass through the placenta to the fetus if the mother was passively immunized with a heterologous immune serum. Fraser, Jukes, Branion, and Halpern (22) showed that the livetin fraction of the yolk of eggs of actively immunized ducks contained antitoxin. The sera of newly hatched ducklings contained antitoxin but it could no longer be demonstrated in ducklings after three weeks of age.

Whether or not antitoxin is present in the colostrum or milk of the human species, analogous to the presence of agglutinins in the colostrum of cows as demonstrated by Smith and Little (23) and Little and Orcutt (24), has been the subject of considerable study with conflicting evidence. Sugg (25) studied the antitoxin content of the serum of a negro woman who had a high natural antitoxin content (1.5 to 2.5 units per cc.) over a long period of time. She was given an injection of toxoid during the second month of pregnancy which resulted in a maximum antitoxin rise to 20 units per cc. This fell to 8 to 10 units per cc. and remained at this level throughout the entire period of lactation. The concentration of antitoxin in the colostrum was about a third of that in the serum. This fell rapidly and remained at about 0.25 per cent of the serum concentration throughout the nursing period. It would appear, then, that diph-

theria antitoxin may be present in the colostrum and milk of the human species, but the experiment quoted above is unusual because of the mother's high degree of natural immunity. There is no convincing evidence that antitoxin in colostrum or milk would be absorbed unchanged from the gastro-intestinal tract of the human infant. It is quite likely to be digested and destroyed.

The evidence is clear that the immunity of the newborn infant to diphtheria is passive, antitoxin having passed the placental barrier from mother to fetus. There is no proof that a "physiological maturation" process plays a role. Furthermore, there is no good evidence that the human fetus may become actively immunized *in utero*. While it is true that this passive immunity of the infant lasts much longer than is usually the case for passive immunization, this can be explained, in part at least, by the fact that the antitoxin is present as a homologous protein. Passive immunity from a homologous protein lasts much longer than from a heterologous protein.

The presence of natural antitoxin in the serum of older individuals in a given population parallels the results obtained by Schick testing. (See below.)

The Schick Reaction. In 1908, Schick (26) carried out intracutaneous reactions with diphtheria toxin directly upon human beings to determine whether or not diphtheria immunity was present. His early experiments were carried out by injecting 0.1 cc. of a 1 : 1000 dilution of toxin intradermally, and the results indicated that a positive intracutaneous reaction suggested an insufficient protection from the disease. A positive reaction is characterized by the appearance in 24 to 48 hours of a slight infiltration surrounded by a red areola 1 to 3 cm. in diameter. The reaction usually reaches its height on the third or fourth day, at which time some necrosis may be present. Following this there is a gradual slow fading, with some desquamation of the overlying skin. The skin at the site is usually pigmented for some time following the fading of the reaction.

The material generally used at the present time consists of a standardized diphtheria toxin, diluted in such a way that 0.1 cc. contains $\frac{1}{50}$ M.L.D. for a guinea pig.

The Permanent Standards Committee of the League of Nations (27) has recommended the following procedures for determining the suitability of toxin for Schick testing; the combining power of the toxin for antitoxin, as well as the toxicity, should be taken into account and only a matured toxin should be used.

This Committee accepts the following alternative definitions of the Schick test dose:

(*a*) The Schick test dose shall be $\frac{1}{10}$ of the minimal lethal dose of the toxin when injection is made in a volume of 0.2 cc. and $\frac{1}{50}$ of the minimal

lethal dose when injection is made in 0.1 cc.; provided that this quantity of the toxin

when mixed with $\frac{1}{150}$ part or more of an international unit of diphtheria antitoxin, and injected intracutaneously into a normal guinea pig, causes no local reaction, but

when mixed with $\frac{1}{1250}$ part or less of an international unit and similarly injected, causes a definite reaction of the type of a "positive" Schick reaction.

(*b*) The Schick test dose, for injection in a volume of 0.2 cc. shall be that quantity of the toxin which,

when mixed with $\frac{1}{150}$ part or more of an international unit of diphtheria antitoxin, and injected intracutaneously into a normal guinea pig, causes no local reaction but

when mixed with $\frac{1}{1250}$ part or less of an international unit and similarly injected, causes a marked reaction of the type of a "positive" Schick reaction;

provided that the toxin is such that $\frac{1}{25}$ of the Schick test dose as above determined, without admixture with antitoxin, when injected intracutaneously into a normal guinea pig, causes a definite local reaction of the type of a "positive" Schick reaction, but that $\frac{1}{50}$ of the Schick test dose, similarly injected without admixture, causes no local reaction of this type.

Taylor and Moloney (28) reinvestigated the League of Nations requirements for standardization of Schick toxin. They found that a toxin with three times the toxicity of the standard gave more easily interpretable reactions. Furthermore, the test for combining power was of little value in detecting the level of antitoxin. It was of value, however, in determining a suitable dilution of toxin because, with this test, smaller differences in concentration of toxin can be detected by the intradermal toxicity test. They advise the use of a fresh rather than an aged toxin, since it contains less toxoid than the latter and thus produces fewer non-interpretable reactions.

The National Institute of Health recommends a number of standard procedures such as sterility tests, safety tests, incubation test (the toxin must withstand incubation for 24 hours at 37° C. without appreciable loss of toxicity), etc., which govern further the manufacture of Schick test toxin.

Formerly Schick outfits were made up with the toxin and its diluent in separate containers. The necessary quantity of toxin was provided in a separate tube or vial and was mixed with the diluent at the time of use. This was necessary because toxin previously diluted with salt solution deteriorated rapidly. This method was obviously cumbersome and subject to several errors such as contamination and inaccuracy of dosage. In 1931, Bunney (29) showed that toxin remained stable under various

adverse conditions if a 2 per cent solution of peptone was used as a diluent. This investigator also stated that Glenny and Waddington had independently used broth as a stabilizing diluent. Later White, Bunney, and Malcolm (30) improved their diluent by using a 0.25 per cent solution of Witte peptone (instead of the "Difco-Proteose" peptone previously used) containing 0.85 per cent sodium chloride and 0.5 per cent phenol. With material prepared with this diluent the reactions following its injection in human beings were entirely comparable with those from freshly diluted toxin. It could be passed through Berkefeld filters and retain its toxicity even after 96 hours' incubation at 37° C. Such material was subjected to various hazards even more severe than those usually met with in the field and in transportation without appreciable loss of potency. Other peptones and similar products may be used as stabilizing agents. At the present time Schick outfits are available ready-diluted, having been prepared with a diluent such as this.

The Pseudo Schick Reaction. Schick (31), in 1913, noticed that a positive reaction was not always proof of the absence of immunity. These false reactions differed from the true ones in several respects: they appeared a little earlier, reached their maximum intensity sooner, and faded more quickly. Park, Zingher, and Serota (32) called such reactions pseudo reactions and felt they were sensitization phenomena. Bessau and Schwenke (33) found that such reactions might follow the injection of heated diphtheria toxin. As a result of this observation a second or control injection was introduced into the Schick test. Furthermore, it soon became apparent to Park, Zingher, and Serota, Zingher (34, 35, 36), and others that immunizing injections often gave rise to unpleasant reactions in those individuals exhibiting pseudo reactions. Thus, the psuedo reaction came to serve two purposes; first, as a control for the Schick test, and secondly, as an indicator of those individuals who might react severely to immunizing injections.

Efforts were soon made to determine what substances were responsible for the production of the pseudo reaction and what immunologic mechanism brought it about. Both Zingher and Zoeller (37) showed that uninoculated broth failed to elicit this response. The causative agent was shown to be heat stable, which eliminated toxin as the cause, since the latter is heat labile. Neutralization of toxin with antitoxin failed to abolish the reaction. It became apparent that the reactive substance was probably present in the bodies of diphtheria bacilli. Park and his associates demonstrated the reaction by using filtered autolysate of diphtheria bacilli. Kolmer and Moshage (38, 39) used a suspension of organisms washed free of toxin, and von Groer and Kassowitz (40) used both a ground suspension of bacilli and the nucleoprotein obtained from them.

The pseudo reaction is regarded, therefore, as a manifestation of hypersensitiveness to diphtheria bacillus protein. It is an example of true bacterial hypersensitiveness in the same sense as the tuberculin reaction. It is highly specific and indicates some previous contact with the diphtheria bacillus. It is absent in infants and becomes more frequent as age advances. It occurs in both susceptibles and immunes but is much more common in the latter.

Other Abnormal Reactions to the Schick Test. A few reports have appeared describing instances of acute anaphylactic shock following the application of the Schick test. Monroe and Volk (41) in collaboration with W. H. Park described two such cases. We quote their own description of their cases:

"March 10, 1932, a boy aged 10, had a Schick test with control. Reading one week later was positive. April 27, the child received the last injection of $\frac{1}{2}$ cc. of toxoid without alum, followed by no local or general reaction. June 2, the child received the final Schick test. One-half hour later the lips and eyelids became swollen, the child became cyanotic and short of breath. Urticaria appeared over the entire body. The attack was relieved by adrenalin.

"A similar attack was observed in a child of three years of age who received the Schick test in the Health Department Office. Within ten minutes the child suddenly developed urticaria all over the body, extreme restlessness, shortness of breath, cyanosis, coughing, vomiting, and swelling of the eyelids and lips. Adrenalin was administered and repeated in the course of the next 15 minutes. The attack lasted over an hour. The history of this child is as follows: July 16, 1932, the preliminary Schick test positive; subsequently he received two 1 cc. injections of concentrated toxoid, the last being given August 23, 1932. Schick tests were given in September and October, 1932, and in February, 1933, all of which were positive. The last which precipitated the attack was given August 12, 1933."

Unfortunately, these investigators failed to state the nature of the diluent in their Schick test material, a question of some importance.

Bousfield (42) described two patients with symptoms similar to, though milder than, those described above, coming on within an hour in one and within 15 minutes in the other, after a Schick test. It is of great importance that one of these patients was proved to be markedly sensitive to Witte peptone, the material used as a stabilizer for the Schick toxin dilution. A 1 to 10,000 dilution of the peptone scratched on the skin gave rise to a definite urticarial reaction.

Fourteen cases of this character have been recorded by Parish (43), seven from his own experience and seven collected from the literature. Two of these cases were known to be sensitive to Witte peptone. This author makes the important observation that such cases do not appear to have been reported prior to 1934 and that an important factor in their

production is the peptone which is now generally employed as a stabilizer in the toxin dilutions. It is important to keep this type of reaction in mind. Additional cases will undoubtedly be seen in the future. If such cases should be seen, it is extremely important that they be carefully studied in order to discover, if possible, what substances the sensitivity is due to. Carefully studied cases of this character should be reported.

The numerous careful experiments of Neill, Sugg, and Richardson (44, 45, 46, 47, 48) should be reviewed in this connection. These investigators showed that typical anaphylaxis could be induced in guinea pigs, using diphtheria toxin or toxoid as the antigen and antitoxin as the antibody in the reaction. They clearly distinguished this reaction from a reaction involving bacterial products other than toxin or toxoid as antigens. We know of no instance of a reaction in human beings wherein this particular mechanism was demonstrated to have been responsible for it. Nevertheless, this type of reaction should be kept in mind, particularly if the antigen should accidently be introduced intravenously at the time of its injection.

The Schick Level of Antitoxin Immunity. Following the acceptance of a $\frac{1}{50}$ M.L.D. for a guinea pig as the Schick test dose, a large number of experiments indicated that this amount of toxin will produce no reaction on intracutaneous injection into a human being if the blood serum of the subject contained more than $\frac{1}{30}$ unit of antitoxin per cubic centimeter. This value, then, has generally been regarded as representing the so-called "Schick level" of immunity. During the past few years a number of reports have appeared which indicate that this "level" is more accurately described by a considerably smaller amount of antitoxin. Glenny and Waddington (49) found that the Schick-negative level in guinea pigs corresponds to $\frac{1}{250}$ unit of antitoxin per cc. of serum. These investigators, as well as Fraser and Halpern (50) and Parish and Wright (51), have shown that the level of Schick immunity in man lies between $\frac{1}{300}$ and $\frac{1}{250}$ of a unit of antitoxin per cc. of serum. We must conclude, therefore, that the old value of $\frac{1}{30}$ unit of antitoxin represents a higher value than that which may be present in many individuals with a negative Schick reaction.

From time to time reports have appeared tending to discredit the Schick test as a measure of diphtheria immunity, largely because of the occasional occurrence of diphtheria in supposedly Schick-negative individuals. While diphtheria may occur, as we shall see later, in individuals with presumably an adequate amount of antitoxin as determined by titration, nevertheless, for all practical purposes the Schick test is an extremely reliable index of immunity. As Zingher (52) pointed out many years ago, certain errors such as improper technique of the test and inac-

curate interpretation of the result may account for many of the discrepancies. Of even greater importance, he studied a number of different brands of Schick testing material in comparison with his own standard toxin. He found extraordinary variation in the potency of these different materials. Brandon and Fraser (53) found considerable variation among different licensed brands of Schick testing material. The most important single factor involved in the accuracy of the Schick test is the use of a product of adequate potency.

The Schick Test and Natural Immunity to Diphtheria. Innumerable studies have been recorded showing the distribution of diphtheria immunity among different populations, under various environmental circumstances, and in various ages. In many instances parallel studies have been made of the antitoxin content of the blood. The following table compiled from figures given by several investigators and representing a very large series of tests illustrates the reaction to the Schick test at various ages.

TABLE II

THE REACTION TO THE SCHICK TEST AT VARIOUS AGES

AGE	PER CENT POSITIVE SCHICK TEST	PER CENT NEGATIVE SCHICK TEST
Newborn	16	84
0–3 months	28	72
3–5 months	43	57
6–7 months	57	43
7–8 months	63	37
8–9 months	84	16
9–10 months	93	7
10–11 months	87	13
11–12 months	91	9
1–3 years	83	17
4–6 years	61	39
6–7 years	50	50
7–8 years	44	56
8–9 years	37	63
9–10 years	32	68
10–11 years	29	71
11–12 years	28	72
12–13 years	24	76
13–14 years	23	77
14–15 years	20	80
15–16 years	18	82
16–17 years	18	82
Over 17 years	14	86

Taken from Schick (54).

Thus it can be seen, as has already been pointed out, that young infants are generally immune to diphtheria. The greatest number of susceptibles occur between the ages of 10 months and 6 years, and it is during this period that diphtheria is most common. After 5 or 6 years the percentage of those with natural immunity increases rapidly.

The percentage of Schick negatives and Schick positives among normal people varies widely according to the environment in which the individuals have lived before they were tested. As a general rule, it may be said that the Schick reaction is positive in a larger percentage of people who have lived in rural communities than in the urban populations and is higher among people who have lived guarded lives, such as the children of the economically upper-classes, than in those who have been freely exposed to contagious contacts in youth. It is likely that in the urban populations and in the less-cared-for groups of the population frequent temporary carrier states are established, perhaps with organisms at a low level of virulence, as a consequence of which a certain amount of diphtheria immunity and antitoxin production results. White (55) has called attention to the relatively higher susceptibility level in the students of private schools and colleges than among the population in general and among the children in public schools. Kelley, Stevens, and Beattie (56) have found a high percentage among rural school children and in colleges from which the student body is recruited from rural communities, as contrasted with the more metropolitan character of the students at the University of California. In rural children the susceptibility as indicated by the test has been as high as 70.1 per cent, and according to White, in Massachusetts among school children in general it was 71.5 per cent. In some communities in which diphtheria had long been absent the susceptibility rate was 100 per cent. Chason (57) found a much higher rate of immunity in children in rural Alabama. He showed that white children who were members of large families had a higher percentage of negative Schick tests than those of small families. Crooks (58), testing nurses and medical students in Chicago, found 69.6 per cent positive and 30.3 per cent negative. Contrasting large groups of people whose residence was rural with an urban group, he found 79.9 per cent positive of the rural as against 50.7 per cent of the urban.

As for populations in different parts of the world, Talliaferro (59) tested a large number of persons in Honduras and found that the white children of American and English parentage between 6 and 14 years of age showed 55.8 per cent positive as compared to 9.6 per cent positive among children from the native schools. Ramon and Nélis (60) studied the immunity of pigmies as well as other natives in the Belgian Congo. They often possessed $\frac{1}{30}$ to $\frac{1}{10}$ of a unit of antitoxin per cc. of serum. The black native children had, on an average, a higher antitoxic titer

than European children of the same age. Cauchi and Smith (61) found a similar high degree of immunity among the natives in Nigeria. Kinneard (62) studied a small group of children in Nassau. Of the colored children aged 1 to 10 years, 90.27 per cent were Schick negative, whereas only 62 per cent of the white children of the same age were Schick negative. In contrast with these results, Wells (63) studied a group of Central and Polar Eskimos. He found a higher rate of Schick positives than is generally found elsewhere. The sera of 58 persons were titrated for antitoxin, 28 contained no antitoxin, and the remainder possessed from $\frac{1}{50}$ to $\frac{1}{2}$ unit per cc. It is the experience of most observers that diphtheria is a much less frequent disease in the tropics than in the more temperate zones.

The question which is most directly pertinent in epidemiological work is whether or not this test can be taken as a reliable index of susceptibility to diphtheria. The studies of Park and Zingher, in particular, indicated that Schick negativeness is satisfactory evidence of sufficient protection against accidental infection. O'Brien, Okell, and Parish (64) investigated the subsequent histories of a Schick-negative group exceeding 20,000 among which there were only 18 who contracted diphtheria, and, in every case but one, the disease was mild, with little toxemia. Underwood (65) has recently published an excellent report of his own experiences as well as a review of the literature. He reported 16 clinical cases of diphtheria (among 20 notified) in a Schick-negative population of 2761 persons. Thirteen of these cases required serum treatment. It may be of some significance that the "gravis" type of organism was isolated from 19 of the 20 notified cases. Titration of the blood antitoxin at the beginning of the illness showed that 5 of 7 should have been immune. This question will be dealt with in more detail subsequently when we discuss artificial active immunization.

The Schick test indicates that a high percentage of individuals beyond the childhood age in any population is immune to diphtheria. It is of some interest to know what the general antitoxin level is in such groups. The majority of people possess a small but adequate amount, *i.e.*, less than 1 unit per cc. of serum. Sugg (66) and his associates studied a representative group of 200 individuals and found that 48 per cent had less than 0.5 unit per cc. These investigators also surveyed a larger group of 5015 adults and found that only 1.2 per cent had as much as 2 units of antitoxin per cc. Neill (67) and his co-workers made observations on adults who possessed large amounts of antitoxin, 2 units or more per cc., over a period varying from 1 to $6\frac{1}{2}$ years. The majority of these individuals maintained their high level for long periods of time and, as the authors pointed out, such levels appeared to be a permanent characteristic of such individuals and not a transitory state.

Individuals who showed a drop in antitoxin level gradually approached a lower level which they maintained for long periods.

A question of considerable importance is that concerning the factors responsible for the natural immunization of populations against diphtheria. Two main theories have been proposed to account for this. The so-called "physiological maturation" process of Hirschfeld (68) is based on the following considerations: the low carrier rate of virulent organisms in a population in relation to a high immunity rate; the presence of high immunity rates in populations such as in the tropics and among certain native tribes where clinically recognizable diphtheria seldom occurs; and the presence of natural immunity in certain animals, such as the horse, that do not have recognizable diphtherial infection.

The theory receiving most support, however, is that natural immunity is due to latent or occult infection, the carrier state, "herd" immunization, etc. In other words, specific antigenic stimulation is provided by actual contact with diphtheria bacilli of varying grades of virulence without clinically recognizable disease being produced. A process of this character is undoubtedly operative in many other infectious diseases such as scarlet fever and poliomyelitis.

Grasset (69, 70, 71) and his collaborators have studied this question among the black races of South Africa. They point out that the conditions under which they live expose them freely to "herd" infection. Kinneard (62) suggests that many tropical lesions such as "veld sore," "desert sore," "Barcos rot," etc., are often associated with the presence of *C. diphtheriae*. Young, Cummings, and Wilson (72) showed that herd immunization was responsible for a rapid reduction of Schick-positive rates at the Michigan Home and Training Schools. A very interesting contribution which would appear to answer the objections to herd immunization as a factor on the basis of the infrequent finding of carriers is that of Kaiser and Lode (73), who took cultures twice a week for two years from the pupils in two different schools. In two such classes in Linz only 30 per cent of the pupils remained persistently free of diphtheria bacilli, and in the third class in Innsbruck only 2 per cent of the children were persistently free. This study also indicates the futility of carrier surveys in which one or relatively few cultures are made.

Many horses possess varying quantities of naturally acquired diphtheria antitoxin in their serum. Glenny (74) reported that of 1350 horses admitted to their laboratories over a period of 17 years about half of them had at most 0.1 unit of antitoxin per cc. He observed that in recent years a smaller percentage of horses were found to have natural immunity than formerly. He pointed out that such horses are now largely recruited from country districts, whereas formerly they were largely recruited from the large cities (cab horses, etc.). Richters (75),

Zurukzoghi and Mündel (76), as well as others, have isolated virulent diphtheria bacilli from the upper respiratory tract of horses. Many investigators have also pointed out that this organism is not uncommonly found in skin lesions and wounds in this animal.

Both Dold and Weigmann (77) and Ramon and Erber (78, 79) have found that many monkeys possess naturally acquired diphtheria antitoxin in their serum. It was also shown that these animals might be carriers of virulent *C. diphtheriae.*

Ramon (80) has published a review of the literature on the mechanisms involved in naturally acquired immunity to diphtheria. He concludes that latent infection with the causative organism of the disease is responsible for naturally acquired immunity.

The Primary and Secondary Stimulus in Artificial and Natural Active Immunity. It has long been known that following the first injection of an immunizing agent, considerable time elapses before circulating antibodies are demonstrable. It may be several months after the immunizing injections before a Schick-negative state is reached. Thus, the response to a primary stimulus is very slow. Once, however, an individual has been immunized, the response to a later or secondary stimulus is very rapid. Glenny and Allen (81) showed that if a rabbit has once possessed diphtheria immunity, a minute amount of toxin can act as a secondary stimulus to bring about a rapid increase in antitoxin in the blood. Indeed, they show that in such rabbits the small amount of toxin present in the Schick dose may stimulate secondary antitoxin production. Parish and Okell (82) showed that children with a positive Schick who had once been Schick negative rapidly produced circulating antitoxin in response to the injection of a very small amount of diphtheria toxin. O'Brien (83) mentioned cases in children where a similar process seems to have taken place. One of these was Schick positive and received the immunizing doses of prophylactic. Twelve weeks later she was Schick negative. Tested sixty weeks after this, she gave a positive result and the blood taken was found to contain less than 0.001 unit of antitoxin per cc. When tested three weeks later, without further injection, she gave a negative Schick and the blood was found to contain between 0.01 and 0.1 unit. The operation of this mechanism is observed clinically in that individuals once successfully immunized often have a mild disease if they contract diphtheria. It has also been demonstrated that such patients exhibit a rapid rise in antitoxin production soon after the onset of infection. Furthermore, it is because of this secondary stimulus type of response that horses already possessing antitoxin in their serum usually give the best results under immunization for antitoxin production.

Apparently, then, once an immunity has existed, it may fade, but the most minute stimulus will arouse powerful antitoxin production, a fact

which, as we have seen in another section of this book, is true also of other antibody formation and is one of the fundamental mechanisms of the so-called "permanent" immunity which follows recovery from certain diseases. Such capacity for "hair-trigger" response is specific.

Active Immunization in Diphtheria. An obvious thought which occurred to all the early workers in diphtheria was the possibility of actively immunizing human beings with diphtheria toxin. This was soon found not to be practical because of the toxicity and the necessity of prolonged and individually controlled procedure necessary for any successful immunization. It could be done but would require a degree of skill and persistence in the individual case which would defeat its practical application. As a consequence various other methods have gradually been developed. We shall consider each of these methods separately because each has an individual usefulness under certain specific circumstances. With the development of these methods the prevention of diphtheria by active immunization now ranks second only to the prevention of smallpox.

Active Immunization with Toxin-Antitoxin Mixtures. Theobald Smith (84, 85) suggested the use of a neutralized mixture of toxin and antitoxin for the preliminary injections in the immunization of horses for antitoxin production. Behring (86) later applied this method in the immunization of human beings. This method represents essentially active immunization with toxin rendered harmless by the addition of antitoxin. Behring explained the immunizing action of such neutral mixtures by the reversible nature of the toxin-antitoxin union as a consequence of which toxin was gradually liberated in the animal body. This supposition has been shown to be, in all probability, correct by the subsequent studies of Hartley (87) upon the antigenic action of toxin-antitoxin precipitates.

The most extensive study of the Behring toxin-antitoxin immunization in diphtheria was made by Park and his associates at the New York City Department of Health.

The directions for the preparation of the mixtures are as follows:

> The toxin and antitoxin used should be at least three months old, from the day of planting and the day of bleeding, respectively. The L_+ dose is determined with reference to the United States standard antitoxin. The unitage of the antitoxin to be used should be determined against this standard antitoxin, so that one unit of the antitoxin to be used, when mixed with the L_+ dose of this toxin, should permit the survival for 96 hours of 25 per cent of the guinea pigs injected. When a tentative mixture of the product has been made, no further toxin should be added. Any changes made should consist, not in the addition of toxin, but in the reduction of toxicity by the addition of antitoxin, if necessary. Approximately one unit of antitoxin is, therefore, added to start with, for each L_+ dose in the bulk

of toxin, and with each addition of antitoxin to the mixture, it shall be immediately thoroughly shaken for 20 minutes. By repeated test and fractional addition of antitoxin, 0.1 of a unit at a time, the mixture is brought to a point where 5 cc. injected into a guinea pig permits survival for 96 hours, and at this point the mixture is ready for filtering. After filtering, the mixture is preserved until at least one half of the guinea pigs injected with 5 cc. doses from each bulk bottle, survive 10 days, but show definite paralysis. The human dose of this was 1.0 cc.

Park, Schroeder, and Zingher (88) gradually changed their mixtures in the direction of less toxicity. They experimented with various mixtures, in all of which there were different amounts of toxin and antitoxin, but all of them striking such a balance between the two that 1 cc. caused paralysis in 300-gram guinea pigs, and 5 cc. caused death in this animal within 10 days. These mixtures were made on the basis of Park's statement that the best mixture is one that is underneutralized, and yet perfectly safe.

Annoying reactions may be obtained because of protein of the diphtheria bacillus found in the toxin. In using mixtures such as those described, in which the balance between toxin and antitoxin was the same, but the actual total amount of toxin was reduced, Park and his associates found that the different preparations gave the same immunizing results, but those having the least amount of toxin and, therefore, the least amount of accompanying bacillus substance, showed the least reaction.

When mixtures employing a 3 L_+ dose or more of toxin were employed, larger amounts of antitoxin were obviously necessary for neutralization, which necessitated, of course, the injection of a larger quantity of horse serum. As a consequence the development of undesirable horse serum sensitivity occasionally followed immunization with these mixtures. In using 0.1 L_+ mixtures, therefore, the amount of horse serum injected was considerably reduced, which resulted in the less frequent development of horse serum hypersensitivity. It should be remembered, however, that the development of this sensitivity is still a rare occurrence even with the use of 0.1 L_+ mixtures.

Accidents due to dissociation of toxin and antitoxin have been reported following the use of mixtures prepared with the larger amounts of toxin. White and Robinson (89) reported such an accident in which severe reactions, typical of diphtheria intoxication, developed in 42 of 54 children who had received an injection of toxin-antitoxin mixture which had suffered a prolonged exposure to unusually low temperatures. These investigators (90) later reported experiments which indicated to them that this phenomenon was largely due to a dissociation of toxin and antitoxin when the mixture was subjected to a prolonged freezing temperature.

The method employing 0.1 L_+ mixtures is the one generally used at the present time whenever the use of toxin-antitoxin mixture is indicated. With this particular mixture, accidents such as that described above are hardly possible, provided the precautions of preparation are observed.

It is an interesting theoretical fact that for the proper test requirements a different proportion of antitoxin is necessary for every particular dose of toxin used. In other words, as stated to us by Dr. E. S. Robinson of the Massachusetts Antitoxin Laboratory, for 3 L_+ dose mixtures, approximately 1.2 units of antitoxin are necessary for each L_+ amount of toxin; for the 1 L_+ mixtures, about 1.05 units of antitoxin are required; and for the 0.1 L_+ mixtures, only about 0.8 unit for every L_+ amount is necessary to give the desired result.

The following are the more important precautions suggested by the National Institute of Health, Washington, D. C., for the preparation of diphtheria toxin-antitoxin mixture:

"The toxin and antitoxin should be of sufficient age to be relatively stable and such stability should be proven before use. To minimize the amount of preliminary testing required, lots of toxin and antitoxin reserved for this purpose should be large enough to permit the use of a single lot of each for a considerable period. No toxin should be used which, when made into a mixture of proper toxicity, does not paralyze guinea pigs receiving the 1.0 cc. dose, as described below in the test for toxicity.

"When a new toxin or antitoxin is introduced for the manufacture of toxin-antitoxin mixtures, the resulting mixture should be tested for stability before adopting for general use. Such mixture should be stored for 30 days at 10° C. and retested for increase or decrease in toxicity, using 5 cc. doses.

"The L_+ dose of the toxin should be determined with reference to the United States standard antitoxin, and the unitage of the antitoxin to be used should be determined against this toxin, so that one unit of the antitoxin to be used, when mixed with the L_+ dose of this toxin, shall permit survival for 96 hours of only 25 per cent of the guinea pigs injected. Great accuracy of the degree sought in standardizing a test toxin is not necessary for this preliminary determination, the final tests on the finished product being more important.

"When a new toxin or antitoxin is to be introduced for routine manufacture of toxin-antitoxin mixture, experimental mixtures should be prepared using varying amounts of antitoxin. It has been found that after the proper proportions of a given antitoxin and toxin have been determined, practically identical mixtures may be prepared from the same lots when the same measuring apparatus is used and the same method of mixing followed.

"The antitoxin should be diluted to contain between 20 and 100 units per cc. and should be added to the concentrated toxin in one operation and the mixture immediately and thoroughly shaken, end over end, for at least five minutes by the clock. This mixture should be added to the chilled diluent containing adequate preservative, and the diluted mixture thoroughly shaken in a mechanical shaker for at least 20 minutes.

"Mixtures should stand for 48 hours at 10° C., or for 24 hours at 20° C., before testing. Additional toxin should not be added to over-neutralized

mixtures. Under-neutralized mixtures should preferably be stored until excess toxicity is lost, but additional diluted antitoxin may be added, the mixture to be thoroughly shaken for 20 minutes in a mechanical shaker immediately after each addition of antitoxin, and allowed to stand for 48 hours at 10° C. before retesting. An over-neutralized and under-neutralized mixture may be combined provided each of the two mixtures approaches the proper toxicity. The temperature of mixtures during preparation should not exceed 22° C. The reaction should be adjusted to *p*H 7.2–7.4. Final filtration to safeguard sterility should take place after this adjustment and before the final test for toxicity.

"The ideal toxicity of diphtheria toxin-antitoxin mixture should be such that when five human doses of the sample (as ready for human use) are injected into each of five guinea pigs weighing 300 grams, two of these 5-dose pigs shall die acutely in less than 10 days and the other three shall die of diphtheria paralysis in from 15 to 35 days. Of five pigs each receiving one human dose, one should die of diphtheria paralysis in from 15 to 35 days, and the other four should be paralyzed, but survive. As permissible variations from this toxicity, guinea pigs may be used weighing from 250 to 350 grams, the weights being scattered through this range for any set of guinea pigs on the same dose. Of the five pigs on five human doses, from four to none may die acutely, more than 50 per cent of the remaining pigs dying of paralysis, and of the five pigs on one human dose, three to none may die of paralysis, some of the remainder surviving with paralysis. Pigs dying with other conditions than diphtheria poisoning (acute or paralytic) shall not be counted."

The value of toxin-antitoxin mixture as an effective immunizing agent cannot be questioned. As Park (91) has shown in summarizing his extensive experience with this agent, three injections of 1 cc. each, spaced at intervals of one to two weeks, will immunize 85 per cent of those to whom it has been administered. Immunity develops slowly following the injection of this agent. It usually takes from one to six months after the last injection for the Schick reaction to become negative.

It was at first thought that toxoid might entirely supplant the use of toxin-antitoxin mixture as an immunizing agent. This has proved not to be the case. As we shall see later, severe reactions are not uncommon during late childhood and in adults following the injection of toxoid, such reactions being due to the proteins of autolyzed diphtheria bacillus cells. For this reason toxin-antitoxin mixture, or similar recently developed products which will be discussed later, should be used for immunization of individuals beyond the age of ten to twelve years.

We shall consider the effectiveness of artificial active immunization in the prevention of diphtheria after discussing the other agents that are used for this purpose.

The Use of Diphtheria Toxoid (Anatoxine) as an Active Immunizing Agent. It had long been known that aging of toxin as well as treating it with certain chemicals resulted in a loss of toxicity. In 1923, Ramon

(92, 93, 94, 95, 96, 97) treated toxin with formalin and incubated it for long periods of time. He found that this resulted in a complete loss of toxicity without appreciable loss of antigenic properties. This investigator applied the name "anatoxine" to this material. In the English literature this product is commonly referred to as "toxoid" and in keeping with this common usage we shall refer to it henceforth by this term.

Toxoid exhibits a number of definite immunologic characteristics. Indeed, it differs very little from unmodified toxin except for the loss of toxicity. As we have already stated, it possesses its original antigenic power, that is, its ability to incite antitoxin production when injected into animals. It is specific in its immunologic reactions. Antitoxin resulting from immunization with toxoid exhibits all the properties and characteristics of antitoxin produced by immunization with unmodified toxin.

As Ramon and others have shown, toxoid flocculates with antitoxin just as does toxin. In fact, Ramon has maintained that the flocculation reaction is an adequate measure of the antigenic value of toxoid. This is true, however, within rather wide limits only. Asakava (98) studied the response in rabbits of different batches of toxoid varying from 7 to 30 in L_f value, and found it almost impossible to distinguish one toxoid from the other by the immunization results. Most authors agree, however, that for immunization purposes a toxoid of high L_f value should be used.

Toxoid is a much more stable product than toxin. Aging does not appear to cause appreciable deterioration of its antigenic value, as Ramon showed by testing samples which had been kept for seven years at laboratory temperature. It is more resistant to heat than toxin. A temperature of 65° to 70° C. does not alter the flocculating or antigenic property of toxoid.

Of great practical importance is the fact that the reaction resulting in the change of toxin to toxoid is not reversible. There is no evidence that completely modified toxin has ever regained any of its toxic properties.

Numerous studies have been made to explain the mechanism by which formalin changes toxin into toxoid. Follensby and Hooker (99), in addition to reporting their own experiments, have reviewed the literature on this subject. They state that "Four hypotheses have been related to the production of toxoid by the action of formaldehyde upon diphtheric toxin: (*a*) the toxin contains an amino-group or groups with which formaldehyde reacts; (*b*) the reaction is a (partial) hydrolysis in which formaldehyde acts as a catalyst; (*c*) combined and not free formaldehyde converts toxin into toxoid; (*d*) formaldehyde causes a reaction of condensation in which two or more molecules of toxin unite to form an atoxic compound." In their own experiments Follensby and Hooker studied the kinetics of the reaction between diphtheria toxin and formaldehyde and found that it was of the monomolecular type. They favored the

theory that the reaction is probably catalyzed with hydroxyl ions and formaldehyde acting as catalysts. As most investigators have pointed out, the true nature of the changes occurring in the detoxifying process will probably not be clearly described until a pure toxin is produced and something of its chemical nature and composition understood. Hopeful progress in this direction is indicated by the studies of Eaton (100, 101, 102) and Pappenheimer (103) on the purification of diphtheria toxin.

In the preparation of toxoid for human use no horse meat should be used in preparing the broth from which the original toxin is made. The toxin should have more than 15 L_f units per cc. The L_+ dose should not be more than 0.2 cc. and the M.L.D. should not be more than 0.0025 cc. The toxin should not have been incubated for more than 10 days. After the desired amount of formalin has been added, the *p*H is determined and NaOH is added, if necessary, to adjust the reaction to *p*H 8.2. The preparation is then incubated for 30 days. At the end of this time and every one to two weeks thereafter preliminary intradermal toxicity tests in guinea pigs may be done. In this test 0.1 and 0.2 cc. amounts of undiluted toxoid are injected and should result in a reaction less than 4 mm. in diameter 48 hours after injection on two successive tests. Schick tests should be done on the guinea pigs at the time of injecting toxoid. If these tests are satisfactory, final standard toxicity and antigenicity tests are done. A preservative is then added to the material. The National Institute of Health requires the following tests for this purpose:

Tests demonstrating detoxification shall be done as follows:

"Five cubic centimeters of crude toxoid given subcutaneously in guinea pigs weighing 300 grams shall cause no signs of diphtheria poisoning, including paralysis, at any time during a period of 30 days. A sufficient number of test animals shall be injected to insure that not less than four shall complete a given test, and animals dying during the 30-day period shall show no signs of diphtheria poisoning at autopsy."

Minimum antigenic requirements for crude toxoid shall be as follows:

"At least ten guinea pigs weighing 270 to 320 grams shall each receive subcutaneously the initial dose of crude toxoid. At the expiration of six weeks each of these pigs shall be injected subcutaneously with 5 M.L.D.'s of a stable diphtheria toxin, the toxicity of which shall be shown by the injection of 1 M.L.D. of toxin into normal guinea pigs weighing 250 grams at the same time. At least 80 per cent of the test animals shall survive for 10 days."

The amount of formalin used in the production of toxoid is important. In general, the period of incubation is shorter the greater the concentration of formalin used. On the other hand, undesirable local reactions may be experienced if too much formalin has been used. The National Institute of Health requires that not more than 0.4 per cent formalin shall be used. Actually, satisfactory toxoid can be made with less formalin, *i.e.*, 0.3 to 0.35 per cent.

The amount of formalin required depends to some extent on the amount of impurities present in the toxoid. Glenny, Hopkins, and Pope

(104) found that the amount of formaldehyde necessary to produce a given effect upon any lot of toxin depends greatly upon its amino-nitrogen content. For instance, a toxin in which the Van Slyke nitrogen was 2.8 mg. per 10 cc. lost its toxicity upon the addition of 0.1 per cent of formaldehyde after 24 hours at 37° C. Another toxin with a Van Slyke figure of 19.6 mg. required 0.3 per cent formaldehyde to produce the same effect. In studies on toxin produced in a simple medium, Pappenheimer and Johnson (105) found that their product was detoxified in 17 days with 0.25 per cent formalin.

For literature dealing with the immunizing value of diphtheria toxoid, the reader is referred to the publications of Ramon and his associates in France, Park and his group in New York, Fraser and his co-workers in Canada, and O'Brien and his associates in England. It is generally agreed that two injections of toxoid, given at intervals of two weeks, will result in a Schick-negative state in about 90 per cent of previously Schick-positive individuals. Three injections will result in reversal of the Schick-positive state in 95 per cent of the cases. Thus, toxoid given in three injections results in a greater percentage of successful immunizations than a similar series of three injections of toxin-antitoxin mixture. Furthermore, the Schick-negative state occurs more rapidly following toxoid than toxin-antitoxin mixture.

At the present time toxoid is generally administered in three doses of 0.5, 1.0, and 1.0 cc. at intervals of two or three weeks. There is considerable evidence to suggest that better immunizing results are obtained when the longer interval between injections is employed. This seems reasonable when one bears in mind the importance of the secondary stimulus as discussed earlier in this chapter. We advise, whenever practical, an interval of three weeks between injections. The value of this type of immunization will be discussed in more detail later.

Toxoid, as we shall see later, often causes severe reactions following its injection in older individuals. For this reason it is preferable to restrict its use to children under twelve years of age unless tests for reactors are carried out (*vide infra*). In communities where diphtheria is present its use should be restricted to an even younger age, *i.e.*, six to eight years.

The "Anatoxi-réaction" of Zoeller (Moloney Reaction). In 1924, Zoeller (106) introduced his so-called "anatoxi-réaction" for the purpose of detecting those individuals who might react unfavorably to immunizing injections of toxoid. His test consisted of the intradermal injection of 0.2 cc. of a 1 : 100 dilution of the toxoid to be used for immunization. Later, Moloney and Fraser (107) employed a similar test, using 0.1 cc. of a 1 : 20 dilution of toxoid, as a substitute for the control in the Schick test. They believed that this material was a better Schick test control and an efficient indicator of possible reactors to immunization. This

test has been most frequently referred to in the literature as the "Moloney Reaction" since O'Brien and Parish (108) applied this term to it.

There has been a good deal of discussion in recent years as to the identity of the Moloney with the pseudo Schick reaction. There has been much discussion, in particular, as to the significance of the two reactions in picking out those individuals who would be liable to get a severe reaction following the injection of toxoid. Mitman (109) has recently presented a critical study dealing with these relationships. He prepared a condensed summary of the epidemiological and clinical literature on this point, together with the results of his own experience.

Mitman is of the opinion that the two reactions are dependent upon the same mechanism and that the Moloney reaction should not be used as a substitute for the control in the Schick test. He feels that a less intense reaction in the psuedo reaction is desirable, since it offers less confusion in diagnosing a positive Schick in the presence of such a reaction. He further states that "the Moloney is not an accurate control of the Schick test because the material cannot be standardized. The diagnosis of a pseudo and negative Schick reaction is made when the reaction in the control arm is of the same type, size, intensity, and duration as that in the test arm; and of a pseudo and positive when there is a significant difference between the two, especially in the duration. Using heated toxin as a control, any appreciable difference in size and intensity in the first few days is significant, because the factor responsible for pseudo reactions is present in equal quantities in the two arms, whereas there is not this equality of content when the Moloney is used as a control."

Others feel that the Moloney test gives definite and useful information. Underwood (110) has published a statistical analysis of results obtained with this test in a large number of individuals of various ages. He used 0.2 cc. of a 1 : 200 dilution of toxoid. He found, in a total of 2666 individuals tested, 519 positive Moloney reactions as compared to only 85 pseudo Schick reactions in the same group and feels that if either test is to be omitted, it should be the Schick control.

We feel that the basic mechanism responsible for both these reactions is the same, namely, bacterial hypersensitiveness. It is likewise clear that more individuals will react to the Moloney test than to the pseudo Schick, although the general trend is the same in both. Individual differences are due, as Mitman has stated, to quantitative differences in the materials used, and this factor seems generally to have been neglected. A simple calculation will make this apparent. The Moloney test, as generally employed, consists of the intradermal injection of 0.1 cc. of a 1 : 100 dilution of toxoid. Diphtheria toxin of good potency will have an M.L.D. of 0.002 cc. A dilution of 1 : 10,000 is necessary for

the production of Schick test material (0.1 cc. to contain $\frac{1}{50}$ M.L.D.). Thus, there is a considerable quantitative difference between a dilution of 1 : 100 and 1 : 10,000. An analogous situation is common knowledge in another instance of bacterial hypersensitiveness. It is not uncommon to find an individual who fails to react to 1 : 10,000 dilution of tuberculin (Mantoux test) but reacts strongly to a 1 : 100 dilution. Differences noted, therefore, between the Moloney and pseudo reactions are not due to the tests but to a difference in the reaction capacity of different individuals to them.

We are of the opinion that the Moloney test should not replace the Schick control in so far as the Schick test is used as a test for immunity or its absence. The Moloney should be used only as a test for possible toxoid reactors if it is desired to immunize older individuals with this material. When immunizing procedures are carried out at an early age, as they should be, reactions to toxoid are very rare, although they have been reported.

Recent investigations make it appear hopeful that a purified diphtheria toxin will be available in the future. If a pure toxin, free of diphtheria bacillus protein, is eventually prepared on a large-scale commercial basis, it is not inconceivable that pseudo reactions will be eliminated. Indeed, Eaton (111) has reported investigations with his purified diphtheria toxin in which he has developed a method for separating diphtheria bacillus protein from it. Furthermore, toxoid prepared from such purified toxin should eliminate many so-called toxoid reactors. This would not only make testing easier but would make it possible to immunize older individuals with toxoid without fear of reactions.

Alum-Precipitated Toxoid. In 1889, Roux and Yersin (112) attempted to purify diphtheria toxin by precipitation with various agents, including aluminium hydroxide, aluminium sulfate, and potash alum. In their studies the precipitated toxin was redissolved in some solvent such as sodium phosphate. Apparently, no effort was made to study the antigenic value of such precipitates until many years later.

Glenny, Pope, Waddington, and Wallace (113) precipitated toxoid with alum and showed that the precipitate gave a better immunization result in horses than plain toxoid. Wallace (114) and Glenny and Barr (115, 116) studied the various factors concerned in the alum precipitation of toxoid. Precipitation occurs throughout a considerable range of different percentages of alum. While different batches of toxoid vary in the amount of alum required for satisfactory precipitation, 1 to 2 per cent alum is usually satisfactory. Greater purification appears to occur when larger amounts of alum are used, but excessive amounts increase the number of severe reactions following its injection. Such precipitates can be redissolved in various solvents such as sodium citrate or sodium

tartrate, thus making it possible to determine the flocculating unitage of the precipitated material. Smith (117) showed that toxoid can be precipitated from solution by salts of most heavy metals having insoluble hydroxides, as well as by some phosphates and various gelatinous precipitates. This investigator suggested that the toxoid is absorbed on to the gelatinous precipitate of the hydroxide.

It has been shown by these workers as well as by Havens and Wells (118) and others that considerable purification of toxoid is effected, especially if the precipitate is washed repeatedly in salt solution. It should be borne in mind, however, that bacillary protein, the substance usually responsible for severe toxoid reactions, is probably precipitated together with the toxoid. For this reason the broth should not be incubated longer than ten days in order to avoid excessive autolysis of the bacilli.

The National Institute of Health requires that toxoid for alum precipitation be produced according to the regulations governing the production of unmodified toxoid together with certain additional regulations described below:

"The finished product shall contain not more than 20 milligrams of alum per human dose, the calculation being based on the total amount of alum added for precipitation."

The following antigenic requirement must be met:

"The human dose when administered subcutaneously to guinea pigs weighing 500 grams shall produce at least two units of antitoxin per cubic centimeter of blood serum at the end of four weeks. At least four guinea pigs shall be used for this test, but the blood serum from all four guinea pigs may be pooled for the test for antitoxic content."

It is generally agreed that the increased antigenic efficiency of alum-precipitated toxoid is due to its being retained in the subcutaneous tissues for a long period of time. It acts as an antigenic stimulus throughout and it is likely that much less of it is removed or destroyed without serving as an antigen than in the case of a soluble preparation such as regular toxoid. Glenny has suggested that, because of its long retention in the tissues, a single dose of alum toxoid can act as its own secondary stimulus.

The retention of alum toxoid in the tissues has been demonstrated in a number of experiments. Faragó (119) removed the tissues at the injection site in guinea pigs at various intervals of time. The tissue was macerated and treated with sodium citrate solution to redissolve the precipitated toxoid. The presence of toxoid was determined by flocculation with antitoxin. It was found that flocculation occurred for three days after injection. Obviously, this method is not suitable for the detection of small amounts of toxoid. Harrison (120) employed a more

sensitive test in making a similar study. He removed the tissue at the site of injection, ground it in a mortar with sand and physiological salt solution, and reinjected the emulsion in normal guinea pigs. It was found that tissues removed as long as seven weeks after inoculation were still able to exert an antigenic effect.

There may be other factors which make alum-precipitated toxoid a more effective immunizing agent. For example, Ramon and others have suggested that the inflammatory tissue reaction at the site of injection is, in itself, of considerable importance.

Alum-precipitated toxoid is generally administered in a single subcutaneous dose of 0.5 or 1.0 cc. Numerous studies have been reported which indicate that a single dose of this material will cause a Schick-positive state to become negative in from 95 to 99 per cent of individuals receiving it. It is thus an extremely effective immunizing agent. It has been well established also that immunity develops more rapidly following the injection of alum toxoid than any of the other immunizing preparations. In fact, 90 per cent of children receiving alum toxoid may be expected to have become Schick negative within two to three months after receiving a single dose.

As in the case of regular toxoid the alum preparation should be used in infants and young children only, because of reactions to diphtheria bacillus protein in older individuals. In our opinion alum toxoid should not be used in individuals older than six years. If it is to be used in older children a preliminary, subcutaneous test injection of 0.05 to 0.1 cc. should be given. If there is no reaction in 24 or 48 hours, the remainder of the dose can be injected. It is better to give the latter injection at least two weeks after the test dose because a better immunization result can then be expected.

The reactions which may occur following the injection of alum toxoid have been something of a drawback in the use of this immunizing agent. A nodule, persisting for several weeks but causing no particular discomfort, is experienced at the site of injection by nearly all children. This can hardly be considered as an unusual or abnormal reaction. Various types of more severe reactions may occur, however. The most frequent of these is a local reaction consisting of redness, induration, tenderness, and pain of varying grades of intensity. Some patients experience general constitutional symptoms with some fever.

The reaction which causes most concern is abscess formation at the site of injection which may rupture and drain spontaneously or may require surgical incision and treatment. It should be noted that the evacuated contents of these abscesses are generally sterile. Scars of varying size often follow the healing of such abscesses. Saunders (121) studied the reactions occurring after the injection of various lots of alum

toxoid. Those preparations containing the largest percentage of alum were responsible for the production of the most severe reactions. Among 16,289 inoculations, Baker and Gill (122) recorded eight with abscess formation. McGinnes, Stebbins, and Hart (123) reported 12 abscesses in 112 individuals who received a particular lot of alum toxoid. Faragó (124) reported a small percentage (0.01 to 0.3 per cent) of abscesses in a large number of immunized children. Shafton (125) immunized 101 children, 25 of whom developed abscesses which were followed by scars in 16. This is, of course, an unusual experience. It must be concluded that this investigator used an inferior alum-toxoid preparation to have encountered such a high incidence of abscesses in his cases. In this connection it is generally advised that if an unusual number of reactions are being observed with a particular lot of alum toxoid its use should be discontinued and it should be returned to the manufacturer.

There are two factors of major importance in the production of reactions following the injection of alum toxoid. One of these is the presence of diphtheria bacillus cell protein giving rise to an allergic reaction in individuals hypersensitive to it, a situation identical with that from regular toxoid. The precipitated alum *per se* appears to act as a foreign body. More reactions due to alum are apt to occur if the concentration of this substance is too great. The amount of alum should be kept as small as possible. There appear to be other factors, however, that are not understood. Two lots of alum toxoid prepared, as far as possible, in an identical manner may exhibit a wide difference in the number and intensity of reactions produced.

A few reports have appeared which suggest that a single dose of alum toxoid may not be as effective as was at first thought. These reports indicate, in particular, that the immunity may not be as lasting as that following the use of multiple injections of other materials. Fraser and Halpern (126) compared the antitoxic immunity (by titration) of a group of children receiving one dose of alum toxoid with another group receiving three doses of unmodified toxoid. They found that, "Of the one dose alum group, at ten weeks, 62 per cent had more than $\frac{1}{100}$ of a unit of antitoxin per cc. of serum; of the three doses unmodified toxoid group, 91 per cent had more than $\frac{1}{100}$ of a unit, ten weeks after the first dose. After one year, only 19 per cent of the alum group remained above the $\frac{1}{100}$ level, whereas 91 per cent of the three dose group remained above that level. If $\frac{1}{50}$ of a unit is taken as the measure of antitoxin response, the figures for the alum group are 30 per cent at ten weeks and 11 per cent at one year, as contrasted with 91 per cent and 69 per cent in the three dose group." Schuhardt and Cook (127) compared the antitoxin response of individuals receiving one dose of alum toxoid with another group receiving two doses of unmodified toxoid. They confirmed the

observations of Fraser and Halpern, finding that even two doses of soluble toxoid resulted in a response superior to that following a single dose of the alum-precipitated material. Pansing and Shaffer (128) found that, in a group of 549 children who were Schick negative 60 days after receiving a single dose of alum toxoid, 57.8 per cent had lost this protection at the end of two years.

It is difficult to understand why immunity should be much less lasting following even a single dose of alum toxoid. It is possible that repeated antigenic stimulation is important in regard to the duration of immunity. The level of antitoxin is higher following multiple secondary stimulation, and there appears to be a direct relationship between the level and the duration of immunity. It must be remembered, however, that, as O'Brien has stated, an individual once successfully immunized can still be considered immune even though the Schick test has returned to a positive reaction because the ability to respond rapidly to a subsequent stimulus is a more or less durable state.

Other Procedures for Active Immunization in Diphtheria. Toxoid-antitoxin mixtures analogous to toxin-antitoxin mixtures are used as immunizing agents to a certain extent, particularly in Great Britain. Since a small amount of toxoid is used in such mixtures, reactions very seldom occur. The immunity response following a series of these injections is about of the same order of magnitude as that following a course of toxin-antitoxin. It is claimed that toxoid-antitoxin mixtures have the advantage of being entirely safe.

It would appear to us to be more reasonable to give the small amount of toxoid present in such a mixture without antitoxin and thus avoid the risk of occasional sensitization to horse serum. If such mixtures fail to give rise to reactions, it must be dependent upon the small amount of toxoid in them. Antitoxin does not neutralize the bacillary protein responsible for reactions. As far as safety is concerned, it is generally agreed that there is little likelihood of danger from dissociated toxin in toxin-antitoxin mixtures if 0.1 L_+ mixtures are employed.

Hartley, in 1925, showed that the washed precipitate or floccules of toxin-antitoxin mixtures made a satisfactory immunizing preparation. Two years later Glenny and Pope (129) prepared similar precipitates from toxoid-antitoxin mixtures, finding them to be as good antigens as toxin-antitoxin floccules. They found further that heating such floccules to 80° C. for one hour increased their antigenic efficiency. Since then toxoid-antitoxin floccules have been used rather extensively as an immunizing agent. It has been reasoned that the specific precipitation of toxoid by antitoxin effects a certain amount of "purification," and reactions are not likely to occur following its use. Again, this material is similar to if not a little less effective than toxin-antitoxin mixtures as an active

immunizing agent. It is generally necessary to employ from four to six injections in order to secure a high percentage of successful immunizations.

Sugg (130) prepared similar toxoid-antitoxin floccules using human antitoxic serum instead of horse antitoxin. He suggested that such material should be useful for the immunization of asthmatics or of persons known to be sensitive to horse serum. This author made the interesting observation that serum made by immunization with floccules had less agglutinins than serum produced by immunizing with crude toxoid, though they were comparable with respect to antitoxin content. This can be taken to indicate that in the preparation of floccules a certain amount of purification actually occurs.

Duration of Artificial Active Immunity. Formerly it was assumed that immunity following artificial active immunization lasted for many years if not for life. There was evidence for believing that it lasted for five to eight years at least. Recent reports suggest that such immunity lasts a much shorter time.

The prolonged immunity previously described is, in all probability, not due to artificial active immunization. Once the child is successfully immunized it possesses the ability to exhibit a maximal response to a minimal secondary stimulus. Even the occasional repetition of the Schick test is often adequate to maintain immunity in such an individual. Thus, the long-continued immunity is undoubtedly due to the factors responsible for natural immunization. In other words, the immunized child receives an occasional inapparent exposure, sufficient to maintain his immunity. Thus, a child not actively immunized, living in the usual urban environment, has about a 50 per cent chance of becoming naturally immune by the time he has reached eight years of age. It is probably more correct to credit the continuance of artificially produced active immunity to the factors responsible for this child's natural immunization.

In this connection we may be permitted to speculate about certain situations which may arise. In a community where diphtheria has long been absent there will naturally be a decrease in carriers. Under these circumstances natural immunization will decrease and greater attention will have to be paid to artificial active immunization, and the latter, as we have said above, may not last long. It may be necessary to repeat the artificial immunization. In the case of a child, immunized at six months, and living in a guarded, upper-class home, in such an environment it would not be unreasonable to advise repeating the Schick test before it enters school at six years of age. If positive, a single injection of one of the accepted preparations should be sufficient to re-establish his immunity.

Recommended Procedures for Active Immunization against Diphtheria. Infants should be immunized with soluble toxoid administered subcutaneously in three doses of 0.5, 1.0, and 1.0 cc. at intervals of two to three weeks between injections. The effect from secondary stimulation is greater when longer intervals between injections are used. The most desirable age for active immunization is between eight and ten months. Passive immunity may last as long as six or seven months in an occasional infant. There is some evidence, as pointed out by Park many years ago, that such residual passive immunity interferes with the development of active immunity. Greengard and Bernstein (131) reported that two thirds of infants with negative Schick reactions at the beginning of immunization had a positive reaction later, whereas the positive reactors turned negative and remained so in 88 per cent of the cases.

It is not necessary to do a Schick test before immunization of infants or children of pre-school age. After this age Schick tests should be done and only the positive reactors immunized. In large-scale immunization programs a post-immunization Schick test is often omitted. This is probably justifiable for economy of time and materials, since 95 per cent of positive reactors will have a negative test two to three months after the last of three injections of soluble toxoid. If 95 per cent of a population is immune, it is unlikely that diphtheria will occur. Nevertheless, whenever practical, it is desirable to repeat the Schick test after immunization, since it is of some importance to the individual to know that immunization was successful.

Soluble toxoid or alum-precipitated toxoid can be used in children up to ten or twelve years of age with little danger of hypersensitive reactions to bacterial protein, particularly if diphtheria has long been absent from the environment. The use of these materials should be restricted to a younger age, *i.e.*, six years, if diphtheria is or recently has been present in the community. They should not be used in individuals with a pseudo reaction to the Schick test. They may be used in older individuals if no reaction occurs after an intradermal injection of 0.1 cc. of a 1 : 100 dilution of toxoid.

Alum-precipitated toxoid may be used in a single dose of 1.0 cc. injected subcutaneously. Elsewhere in this chapter it was indicated that immunity may not last long after a single injection of alum toxoid. Park (132), after reviewing this question in connection with his own work, suggests that it is better to follow a more conservative plan and give either two doses of alum toxoid or three doses of soluble toxoid. We consider the latter procedure the method of choice. The same limitations with regard to age, hypersensitivity to bacterial protein, etc., are involved in the use of alum toxoid as in the use of soluble toxoid. It

should be pointed out again that a nodule persists for some time at the site of injection of alum toxoid, and in some individuals this may result in abscess formation.

All Schick-positive individuals over twelve years of age should be immunized by three subcutaneous injections (1.0 cc. each) of a properly prepared and standardized toxin-antitoxin mixture. Since this results in the reversal of the Schick-positive state in 75 to 80 per cent of susceptible individuals, Schick tests should always be done three months after the immunization. A second course of injections should be given to those who are still positive.

The Value of Artificial Active Immunization in the Prevention of Diphtheria. There have been many studies published which indicate beyond any doubt the value of active immunization in the prevention of diphtheria. The early studies of Park, Zingher, and others in New York City are classical demonstrations of this. The study of McKinnon and Ross (133) in Toronto is a striking demonstration of the effect of immunization. For over 30 years there had been a fairly constant morbidity rate in this city, and in 1926 and 1927 the rates were 16.2 and 20 per hundred thousand. In 1934, in this city of 630,000 people there were only 18 cases of diphtheria and there had been no deaths from this disease for a period of 15 months. Reh (134) has reported a striking fall in the diphtheria rates in the canton of Geneva following the widespread use of active immunization since 1929.

Diphtheria is always a troublesome problem in children's preventoria, convalescent homes, etc. These institutions, therefore, provide an ideal situation for the study of the effectiveness of active immunization and, indeed, many such studies have been reported. Parisot, Melnott, and Engel (135) have reported their experiences at the Flavigny Preventorium. At the beginning of their study in 1927 the morbidity rate was 10.32 per cent. Since April, 1928, all susceptible children admitted were immunized with toxoid, resulting in a marked drop in the number of cases. From April, 1934, to September, 1935, there were only six cases of diphtheria among 1275 admissions, a morbidity rate of 0.47 per cent. Dudley, May, and O'Flynn's (136) report on similar observations should be read in full.

In this discussion we have not intended to give the impression that either natural or artificial active immunity is absolute assurance against the occurrence of diphtheria, for such is not the case. The physician should never be lulled into such a false sense of security as to regard a membranous inflammation in a child's throat as not being diphtheric because such a child had been actively immunized and exhibited a negative reaction to the Schick test. Diphtheria can and does occur, though rarely, in immune individuals, particularly under circumstances such

that exposure to virulent infection is very heavy. Diphtheria has occured with disheartening frequency in districts such as Leeds, England, where the gravis type of infection is particularly prevalent. In this connection, however, we should refer again to our previous discussion, in which it was pointed out that the disease is usually milder in the immunized patient and that such a patient has the ability to respond rapidly with his own antitoxin production.

Production, Processing, and Standardization of Diphtheria Antitoxin. In addition to the usual procedures for selecting horses of sound health, it is important to select and use those animals whose serum contains some natural diphtheria antitoxin, since they respond much more rapidly with increased antitoxin production during the course of immunization.

In the past, the first immunizing injections consisted of toxin-antitoxin mixtures, followed by small doses of toxin which were gradually increased throughout the period of immunization. Toxoid has now completely replaced toxin-antitoxin mixture, and this agent is frequently employed throughout the entire period of immunization.

The immunization procedure employed for new horses by Dr. Elliott S. Robinson * of the Massachusetts State Antitoxin Laboratory is as follows:

The injections of toxoid are given subcutaneously, the first dose being 5.0 cc. Successive injections are given on opposite sides of the horse at intervals usually of two or three days. The doses are increased by 10 or 20 cc. at each injection until 200 cc. is reached. Then toxoid plus calcium chloride is substituted, starting with 50 cc. and increasing by 10 cc. at each injection until the horse responds with a temperature reaction of 38.5 to 39.5° C. The temperature should return to normal the day before the next injection. If it has not returned to normal, the next injection is omitted. The maximum dose is 200 cc. of toxoid plus calcium chloride. The maximum injected at a single site is 60 cc. of toxoid alone or 30 cc. of toxoid with calcium chloride. To prepare toxoid plus calcium chloride, to each liter of toxoid add 25 cc. of a sterile solution which contains $\frac{1}{3}$ gram of calcium chloride per cc.

The first bleeding of 7 to 8 liters is usually taken on about the 25th day of immunization. Later bleedings of 9500 cc. are taken into 525 cc. of 10 per cent sodium citrate. At each bleeding about 75 cc. are taken into a small bottle and allowed to clot, to give serum for Ramon and Ehrlich tests. The bleedings are discarded unless the test serum shows a titer of 400 units or more per cc.

The routine schedule for producing horses is to give three injections of toxoid plus calcium chloride at intervals of three days. After a rest of seven days following the third injection a test bleeding and a bleeding of 9.5 liters is made. The next injection series is started the following day. When the titer of the serum falls below 400 units per cc., the horse is taken off production.

* We are indebted to Dr. Robinson for this and other descriptions of standard procedures described in this chapter.

If a horse gets too much local or general reaction from injections, doses may be omitted or increased more slowly than the usual schedule. The number of injections given is considered more important than the size of the dose.

Since diphtheria antitoxin is contained largely in the pseudoglobulin fraction of horse serum, concentration of the antitoxin is effected by the precipitation of this fraction. The precipitated pseudoglobulin is freed from salts by dialysis. The methods of concentration employed at the present time are based upon those developed by Gibson and by Banzhaf (137, 138, 139, 140, 141, 142, 143).

The following technique is employed by Dr. Robinson of the Massachusetts State Antitoxin Laboratory:

The citrated plasma is allowed to sediment in the cold for 48 hours, after which the supernatant plasma is drawn off. If the plasma is not to be concentrated immediately, it can be kept in cold storage for a not too long period; later there is a tendency for the plasma to jellify. If cold storage is not available, it can be preserved by the addition of 0.35 per cent Trikresol mixed with an equal volume of ether to prevent precipitation.

A saturated solution of ammonium sulfate is used for the purpose of removing fibrin and euglobulin. The plasma, selected from horses showing approximately the same antitoxin strength, is mixed. If no Trikresol has been previously added, add 0.7 per cent of a mixture of equal parts of ether and Trikresol. A 25 cc. portion is retained for flocculation tests and a 100 cc. portion is used in determining the amount of ammonium sulfate to be used for removing fibrin and euglobulin. The latter is carried out as follows:

To the 100 cc. of plasma, 50 cc. of water is added together with enough saturated ammonium sulfate solution (*p*H 5.4–5.6) to bring the concentration to 28 per cent. The mixture is heated to 57° C. for 1 hour and 25 minutes and raised to 63° C. in 5 minutes. It is then cooled and filtered. The filtrate is diluted with an equal volume of physiological salt solution, then saturated with dry sodium chloride. If too heavy turbidity appears, it is evident that all the euglobulin is not precipitated. The test is performed again, raising the ammonium sulfate saturation to 29 per cent and so on up to 32 per cent if necessary. The amount necessary for the total volume of plasma can be calculated. For plasma containing 400 to 500 units per cc., 30 per cent saturation is usually satisfactory.

Saturation at 28 to 32 per cent precipitates the fibrin and euglobulin. After adding ammonium sulfate solution, the mixture is heated to 57° C. as rapidly as possible in a steam-heated glass-lined kettle and held at this temperature for 1 hour and 25 minutes, and then heated as rapidly as possible to 63° C., after which it is cooled immediately to room temperature and filtered.

The filter papers with the precipitate are thrown into crocks and macerated in a volume of water equal to one fourth the amount of the plasma originally used. After thorough mixing to a homogenous suspension, saturated ammonium sulfate solution is added to a concentration representing 2 per cent above the concentration used in producing the first precipitate. The mixture is then filtered and the filtrate combined with

the first filtrate. The filters and precipitates after thorough draining are pressed between muslin cloths to remove all the liquid. The filtrate should be water clear.

The second precipitation is carried out as follows:

To the measured filtrate obtained from the 28 to 32 per cent saturation and from the precipitate washings, enough saturated solution of ammonium sulfate is added to bring the concentration of the mixture to 50 per cent saturation. It is stirred well and allowed to stand overnight, and then filtered on hardened filter papers. The first runnings, if cloudy, are run through the papers again. All the funnels are covered with filter paper and kept moist in order to prevent crystallization of the ammonium sulfate. Forty-eight hours is usually allowed for filtration. In the 50 per cent saturation all the pseudoglobulin (which contains the antitoxin) is precipitated, while the albumin and other proteins remain in solution.

To obtain a purer product, the pseudoglobulin may be reprecipitated. For this the material is thoroughly drained, the precipitate redissolved in salt solution equivalent to the volume of original plasma, and to the final measured solution an equal volume of saturated solution of ammonium sulfate is added. This will give a 50 per cent saturation. It is filtered as before on hardened paper and allowed to drain. When thoroughly drained, the papers are removed with the precipitate and laid on coarse filter papers to absorb the fluid, using about 12 sheets of 20 × 20 inch filter paper. Any crystallized salt on the edge of the paper is scraped off and discarded. Then the precipitate is scraped away about four inches from the edge of the paper all the way around. The precipitate is smoothed off into an even cake and when sufficiently firm, two precipitates are placed together, "butter" side to "butter" side. These are placed between dry, coarse filter papers to make a pile of not more than six precipitates and put in a press and pressed gently. The papers are changed frequently, at first changing the filter papers as soon as moist, and after each change the pressure is gradually increased. The precipitates should not be too thick and any that are thin are combined. For 100 liters of plasma the final pressing is carried on with twelve precipitates. Pressing is continued until the filter papers absorb no more water.

For the dialysis any crystallized salt from the filter papers is removed. The precipitate is broken into small particles and placed in bags of #300 cellophane which have been previously filled with water and examined for leaks. They are placed in a dialyzing tank with running water and dialyzed until just a trace of sulfate shows as tested with barium chloride. The time of dialysis depends on the temperature of the water and the amount of the precipitate put into the bag. For a bag containing three and a half pounds of antitoxin, it requires 36 to 48 hours, when the temperature of the water is about 21° C.

When dialysis is finished, the bags are punctured, the contents measured, 0.9 per cent sodium chloride added to make the antitoxin isotonic, and 0.35 per cent Trikresol is added as a preservative. (The Trikresol is first emulsified with physiological salt solution, in the proportion of 1.0 cc. of Trikresol to 9.0 cc. of salt solution.) At the end of four months or later, the antitoxin is filtered through paper pulp in a Buchner funnel and then through a Berkefeld. The total solids and ash are then determined. The finished product should have a total solids of not more than 20 per cent, ash of not more than 1.0 per cent, and a *p*H of 5.8 to 6.

Before release for clinical use sterility tests and antitoxin content are determined.

The Standardization of Diphtheria Antitoxin. Ehrlich method. The method of Ehrlich, in which an unknown horse serum in dilution is measured in 250-gram guinea pigs by subcutaneous injection against an L_+ dose of toxin is still the one relied upon for the final determination of the number of units of antitoxin per cc. of antiserum. Standard antitoxin for this purpose is provided by the National Institute of Health. There are a great many technical specifications which have been standardized in order to produce uniform and accurate results. These were thoroughly established by Ehrlich, and have been adapted and carefully described for the United States by Rosenau (144). The reader is referred to his publication for a thorough discussion of methods and sources of error.

In the standardization of antitoxin according to the principles laid down in our chapter on the toxin-antitoxin reaction, preliminary measurements of toxin for the determination of M.L.D. and L_+ doses are made. Examples of these measurements, as taken from Rosenau's description, are given in the following tables:

TABLE III

DETERMINATION OF THE M.L.D. OF TOXIN

Dose in Cc.		*Result*
0.03	=	death in 1½ days
0.02	=	death in 1½ days
0.01	=	death in 2 days
0.008	=	death in 3 days
0.006	=	death in 3½ days
0.005	=	death in 4 days = **M.L.D.**
0.004	=	death in 6 days
0.003	=	death in 8 days
0.002	=	late paralysis
0.001	=	well in 16 days

The M.L.D. is defined as that amount of toxin which, when injected subcutaneously, causes the death of a 250-gram guinea pig in four days.

TABLE IV

DETERMINATION OF THE L_+ DOSE OF TOXIN

The "standard" antitoxin is used in this measurement.

1 antitoxin unit + 0.2 cc. toxin = 0
1 antitoxin unit + 0.21 cc. toxin = 0 = L_0
1 antitoxin unit + 0.22 cc. toxin = local infiltration
1 antitoxin unit + 0.23 cc. toxin = fatal in 17 days
1 antitoxin unit + 0.24 cc. toxin = fatal in 14 days
1 antitoxin unit + 0.26 cc. toxin = fatal in 9 days
1 antitoxin unit + 0.28 cc. toxin = fatal in 6 days
1 antitoxin unit + 0.29 cc. toxin = fatal in 4 days = L_+
1 antitoxin unit + 0.3 cc. toxin = fatal in 3 days

The L_0 dose of toxin is defined as that amount of toxin which when mixed with one standard unit of antitoxin just fails to cause a reaction when injected subcutaneously in a 250-gram guinea pig.

The L_+ dose is that amount of toxin which when mixed with one standard unit of antitoxin causes the death of a 250-gram guinea pig in four days after its subcutaneous injection.

The actual determination of the antitoxin unitage of an unknown serum is made by the injection of mixtures containing, each, an L_+ dose and fractions of the serum, mixed together in syringes, in a total volume of 4 cc. each and injected subcutaneously into 250-gram guinea pigs just above Poupart's ligament. An example of a standardization by the Ehrlich method is given in the following table:

TABLE V

THE STANDARDIZATION OF AN UNKNOWN SERUM

1 L_+ (0.29 cc. toxin) + $\frac{1}{500}$ cc. of antitoxic serum	= lives
1 L_+ (0.29 cc. toxin) + $\frac{1}{600}$ cc. of antitoxic serum	= lives
1 L_+ (0.29 cc. toxin) + $\frac{1}{700}$ cc. of antitoxic serum	= lives
1 L_+ (0.29 cc. toxin) + $\frac{1}{800}$ cc. of antitoxic serum	= dies in 8 days
1 L_+ (0.29 cc. toxin) + $\frac{1}{900}$ cc. of antitoxic serum	= dies in 4 days
1 L_+ (0.29 cc. toxin) + $\frac{1}{1000}$ cc. of antitoxic serum	= dies in 2 days

In the above experiment according to the original Ehrlich standard the serum would contain 900 units of antitoxin to the cc. In order to allow a margin of safety, however, Rosenau and others have suggested that the actual unitage with which an antitoxin is labeled should be determined not by the quantity of antitoxin which delays death by the L_+ dose for four days but rather by the quantity which, together with the L_+ dose, results in a survival of the guinea pig. On that basis, the above serum would be labeled as containing 700 units per cc.

We have given merely the skeleton outline of the principles of the Ehrlich standardization. There are many precautions as to the method of making dilutions, details of injection, etc., which are important for accuracy but can be learned only by actually performing the tests.

Standardization by Flocculation. A distinct addition to standardization as well as to theoretical conceptions of the toxin-antitoxin reaction was made by Ramon (145, 146, 147), who demonstrated in 1922 that by a special technique toxin and antitoxin could be made to precipitate each other in proportions which depended upon the relative strength of the two reacting constituents, thus furnishing a further method for the preliminary titration of the antitoxin values of any given serum. Calmette had long before this shown that snake venom and antivenin would precipitate each other, but the method had not been extended to other toxin antigens nor had it attained any practical importance. Useful standardization methods depending upon precipitation have been developed particularly by Ramon himself and by the workers in the Wellcome Laboratories in England, particularly by Glenny and Okell (148).

The exact technique employed depends upon the materials being tested. For testing sera containing 100 to 2000 units per cc., it is convenient to use a toxin (or toxoid) containing 10 to 20 L_f per cc. Using a toxin with 15 L_f per cc. for testing a serum thought to have about 700 units per cc., a serum dilution of 1 : 5 is made. 0.07, 0.08, ... 0.15 cc. of the dilution is placed into a series of small tubes of similar calibre, each of which receives 0.01 cc. more of the serum dilution than the tube before it. To each tube is added 1.0 cc. of toxin and the contents mixed by inverting each tube three times. The tubes are then placed in an incubator or water bath and observed at intervals to note the first tube in which flocculation occurs. With whole horse serum, this is usually within ten minutes to an hour if the tubes are in a 41°C. water bath.

Calculation of the potency of the serum is simple. If the first flocculation took place in the example cited in the tube containing 0.12 cc. of the serum dilution, then 0.024 cc. of the whole serum (0.12 cc. of a 1 : 5 dilution) neutralized 15 L_f of toxin, and 1 cc. (41.6 × 0.024 cc.) will neutralize 41.6 × 15 L_f, or 624 L_f. The serum therefore contains 624 units of antitoxin per cc.

The Ramon test corresponds roughly to the unitage obtained by the Ehrlich method and is used in many laboratories in preliminary titration, but it has not displaced the Ehrlich method, largely because the latter represents the actual value of the serum to save life in the presence of the given dose of toxin and therefore is more directly indicative of therapeutic value. The flocculation reaction is of great value, as we have indicated elsewhere in this chapter, for the standardization of both toxoid and alum-precipitated toxoid.

Intracutaneous Methods of Determining Toxin and Antitoxin Values. Römer (149, 150, 151) and his associates developed a method of diphtheria antitoxin standardization which depends upon intracutaneous injections into guinea pigs. The principle of this test consists in the observation that, when very slight amounts of diphtheria toxin are injected intracutaneously into the abdominal skin of guinea pigs, small areas of local necrosis result within about 48 hours. When such injections are made with mixtures of toxin and antitoxin, the presence of free toxin is indicated by the appearance of necrosis.

Before proceeding to the standardization by this method it is necessary to determine the "limes-necrosis" (just as Ehrlich determined his L_+ dose), that is, the amount of toxin which, together with a given amount of antitoxin ($\frac{1}{50}$, $\frac{1}{200}$, $\frac{1}{2000}$ unit), will still produce a minimal amount of necrosis after intracutaneous injection into guinea pigs. It is necessary, therefore, arbitrarily to choose a definite fraction of an antitoxin unit and mix this with varying amounts of toxin and inject the mixtures into guinea pigs intracutaneously. Those mixtures in which the toxin is completely neutralized will give rise to no lesion further than, possibly, a slight local edema. Those in which there is a large excess of toxin

will cause extensive necrosis. Between the two, in the series, there will be a mixture in which slight local necrosis results from the injection. In this mixture the amount of toxin, just sufficient to cause noticeable necrosis in spite of admixture with the antitoxin, contains the L_n (limes-necrosis) dose.

The methods most commonly applied are those of Römer and that described by Glenny and Allen (152). In all these methods a determination is made of the smallest amount of toxin which still gives a noticeable skin reaction when mixed with $\frac{1}{300}$ to $\frac{1}{500}$ standard antitoxin unit. This dose of toxin is then measured by intracutaneous injection into white guinea pigs after mixing with varying amounts of the antitoxic serum to be tested. When such a mixture, made up in 0.2 cc., produces a skin reaction equivalent to that given by the same toxin mixed with $\frac{1}{500}$ unit of the standard antitoxin, the unknown serum is assumed to contain $\frac{1}{500}$ units.

In 1924, Fraser and Wigham (153) adapted the intracutaneous method to use in rabbits instead of guinea pigs and since then the rabbit method has largely replaced the older method. Because of the larger area of skin available, a greater number of tests can be done on a single animal. Furthermore, the rabbit appears to be more sensitive to diphtheria toxin, weight for weight, than the guinea pig.

The following description of the rabbit intradermal technique was prepared for us by Dr. Elliott S. Robinson.

All dilutions are made with physiological salt solution, measured with a standard 100 cc. biuret. Volumetric pipettes calibrated "to contain" are used in making serum and toxin dilutions, and washed out as in the subcutaneous guinea pig method. A specially calibrated "to contain" pipette is used for measuring the standard antitoxin. Ordinary serological 1.0 cc. pipettes are used for measuring the final dilutions into tubes for the toxin-antitoxin mixtures.

Diphtheria toxin with a known L_+ is diluted so as to contain about $\frac{1}{12}$ L_+ per cc. The sera are diluted so as to cover the probable potency range of the serum. One cubic centimeter of the serum dilution is added. The toxin and serum dilution is mixed thoroughly by inverting the tubes three times. Then 0.1 cc. of the mixture is injected intradermally into the rabbit's skin.

Various doses of a standard diphtheria antitoxin are injected, to permit any correction necessary for the toxin used in the test or for differences in susceptibility of the animals.

The reactions are read 66 to 72 hours after injection. The potency of a serum is determined by the dilutions of serum and standard antitoxin which fail to give a reaction and by the highest dilution of serum which gives a lesser reaction than one of the standard antitoxin dilutions. At least two rabbits are used in each test, and the test repeated if discordant results are obtained or if no end point is reached on one or both rabbits.

Rabbits of the same breed are preferred. Chinchillas seem to be more reactive than the New Zealand whites.

TABLE VI

SCHEME FOR DILUTING TOXINS AND SERUMS

Toxin Dilutions	*Standard Antitoxin Dilutions*
Toxin 1 cc. + Saline 9 cc. (Dil. A)	S. A. 0.66 cc. + Saline 19.34 cc. (Dil. A)
Dil. A 4 cc. + Saline 76 cc. (Dil. B)	Dil. A 1.8 cc. + Saline 2.2 cc. (Dil. B)
Dil. B has + $\frac{1}{12}$ L_+ per cc.	2.0 cc. + Saline 2.0 cc. (Dil. C)
	2.2 cc. + Saline 1.8 cc. (Dil. D)
	2.4 cc. + Saline 1.6 cc. (Dil. E)
	2.6 cc. + Saline 1.4 cc. (Dil. F)
	2.8 cc. + Saline 1.2 cc. (Dil. G)

Typical Serum Dilution	*Mixtures for Injection Contain*
Serum 1 cc. + Saline 19 cc. (Dil. A)	Toxin Dilution B, and
Dil. A 1 cc. + Saline 9 cc. (Dil. B)	Standard Antitoxin Dilutions, C,
Dil. B 1 cc. + Saline 10 cc. (Dil. C)	D, E, F, or G;
Dil. B 1 cc. + Saline 11 cc. (Dil. D)	or Serum Dilutions, C, D, or E
Dil. B 1 cc. + Saline 12 cc. (Dil. E)	

Intracutaneous methods of determining toxin and antitoxin strength are particularly useful since they economize material and animals and can be done with considerable accuracy. They have been extensively employed in investigations in which it is desired to determine conditions in human blood, milk, or other secretions when only a small amount of the material is available. They are extremely valuable for testing for very small amounts of toxin or antitoxin. Glenny and Llewellyn-Jones (154) stated that by this method it is possible to determine the presence of as little as $\frac{1}{50000}$ unit of antitoxin in 0.1 cc. of serum. Furthermore, the presence of circulating toxin in patients with diphtheria can be determined by intracutaneous methods. A study by Harriehausen and Wirth (155) illustrates the results obtained in such tests. Normal human serum injected intracutaneously in guinea pigs never caused necrosis. Neither did the similar injections of the sera of children suffering from varicella and other diseases. Of twelve children suffering from diphtheria, however, serum taken before the administration of antitoxin caused necrosis upon intracutaneous injection into guinea pigs, in every case. In spite of the administration of antitoxin, toxin was demonstrable in the blood in five cases as long as the 35th day. Of ten cases of post-diphtheritic paralysis, toxin was demonstrated in the blood of five.

There are several phenomena concerned in toxin-antitoxin reactions of considerable importance from the point of view of standardization of sera, such as avidity and the Danysz phenomenon. Early investigators began to doubt whether the value of a serum as determined by the Ehrlich method of standardization was a true measure of its therapeutic value. It gradually became apparent that certain qualitative, as well as quantitative, differences existed between sera which were described

by the term "avidity." With the development of Ramon's flocculation test it became apparent that certain sera flocculated with toxin much more rapidly than others. Ramon concluded that the rate of flocculation was a measure of the avidity of a particular antitoxin. It is generally agreed, however, that the speed of flocculation also depends on other factors than avidity. This question has been extensively investigated by Glenny, Pope, and Waddington (156).

The avidity of antitoxin has been demonstrated particularly by Madsen and Schmidt (157), who showed that an L_0 mixture prepared with a rapidly flocculating antitoxin was nontoxic when injected intravenously in rabbits immediately after mixing, whereas a similar mixture prepared from a slowly flocculating serum was toxic for as long as fourteen hours after mixing. Locke, Main, and Miller (158) have described an avidity unit in terms of the amount of antitoxin which neutralizes one L_0 dose of the toxin five minutes after mixing, as determined by the intravenous injection in rabbits.

While the phenomenon of avidity is of considerable theoretical interest in connection with the mechanism of toxin and antitoxin combination and is of practical importance in the standardization of sera, it is not regarded as a problem of great concern in the practical therapy of patients with diphtheria, since antitoxin is practically always administered in great excess.

Passive Immunization and the Treatment of Diphtheria with Antitoxin. The treatment of diphtheria with antitoxin is one of the most conspicuous successes in the entire field of serum therapy. As a result the mortality has been reduced to a low figure. It should be pointed out, however, that the mortality still fluctuates between 6 and 10 per cent, a figure that is still too high. The one factor still responsible for too many deaths is late diagnosis, a factor which cannot in all cases be blamed on the physician. All too frequently diphtheria in a child is considered simply as a cold or sore throat for several days by the parents. The importance of early diagnosis and the promptness of antitoxin administration will be dealt with more fully later.

The dosages of antitoxin recommended by Schick and to which we subscribe are as follows:

For passive immunization in heavily exposed susceptible individuals = 25 units per pound of body weight.

In mild and localized diphtheria = 50 units per pound of body weight.

In severe and serious cases = 250 units per pound of body weight.

Although the injection of antitoxin is of benefit by whatever route it may be injected, nevertheless experimental investigations have taught us much regarding the proper use of this therapeutic agent. There is a great difference in therapeutic efficiency, according to the method by

which the serum is administered, a difference probably depending upon the speed of absorption. The work of Schick, of J. Henderson Smith (159), and of Park and Biggs (160) has thrown much light upon the speed with which injected antitoxin may be expected to become effective. Smith measured the speed of absorption of antitoxin injected subcutaneously into the abdominal wall of a healthy man. His results are shown in the following table:

TABLE VII

THE SPEED OF ABSORPTION OF ANTITOXIN WHEN INJECTED SUBCUTANEOUSLY

One cc. of patient's serum contained:	
Before injection	No demonstrable antitoxin
5 hours after injection	0.1 unit of antitoxin
14 hours after injection	0.225 unit of antitoxin
32 hours after injection	0.68 unit of antitoxin
44 hours after injection	1.0 unit of antitoxin
3 days after injection	1.3 units of antitoxin
4 days after injection	1.3 units of antitoxin
6 days after injection	0.68 unit of antitoxin
13 days after injection	0.17 unit of antitoxin
15 days after injection	0.14 unit of antitoxin
20 days after injection	0.08 unit of antitoxin
27 days after injection	No demonstrable antitoxin

Park and Biggs have made similar studies and have contrasted the speed of absorption after subcutaneous administration with that after intravenous injection. Park (161) also measured the speed with which antitoxin appears in the blood after subcutaneous and intramuscular injection by administering 10,000 units of antitoxin to a 25-pound child. When the serum was given subcutaneously, there were, after 12 hours, 1.5 units per cc. of blood; when given intramuscularly, there were in 12 hours 4.5 units; intravenously administered into a child of the same weight, there were immediately 12 units per cc. After 48 hours the intramuscularly injected child had 4 units per cc. and the intravenously injected one 8 units. It is apparent from this work that antitoxin, subcutaneously given, is slowly absorbed, and does not reach its maximum concentration in the blood stream until 48 hours or more after the injection. During this time also there will have been a loss of antitoxin. As a net result, the maximum concentration in the blood stream will never be as great, following subcutaneous injection, as that after the injection of the same amount intravenously. Furthermore, it is more rational to inject a single adequate dose than to divide the dosage and inject at intervals.

It is thus quite apparent that the maximum effect of antitoxin is secured in the shortest time when it is given intravenously. Antitoxin should be given by this route to seriously sick patients in whom the

disease is running a rapid and fulminating course. Since serum causes both more frequent and more severe reactions when given in this manner, it is desirable to use the intramuscular route in milder cases where speed is not so urgent.

A factor of great practical importance in diphtheria is the rapid and firm fixation of toxin to the tissues as we have discussed in an earlier part of this chapter. Many experiments have illustrated this and we already described those of Glenny and Hopkins. These facts make it urgent that a patient receive antitoxin as soon as possible. Once a lethal dose or more of toxin has been fixed by the tissues, enormous doses of antitoxin will not save the individual. In other words, then, the actual therapy of a case is in a sense prophylactic. The patient must receive treatment before a lethal dose has been fixed by the tissues. For this reason, also, there is no logic in using the enormous doses of antitoxin that are sometimes advised. The sole purpose of antitoxin therapy is to neutralize such toxin as has not already become fixed by the tissues as well as that which will continue to be formed in the lesion.

The Use of Diphtheria Antitoxin as a Prophylactic Agent. In the past diphtheria antitoxin was used much more extensively as a prophylactic agent than at the present time. Its use for this purpose should be restricted to heavily exposed, known susceptible individuals, particularly young children, in whom an attack of diphtheria would be calamitous for reasons of other physical disability and where supervision is inadequate. The indiscriminate use of antitoxin as a prophylactic, particularly when the exposed individual is under adequate medical supervision, cannot be condoned. We recommend that in severely exposed individuals, especially children, the wisdom of prophylactic antitoxin administration be determined on the basis of an immediate nose and throat culture and temperature, all of which can be done within 24 hours. Only a definitely exposed child with a positive Schick reaction and a positive nose and throat culture should be considered a candidate for prophylactic antitoxin. Under these circumstances it is not necessary to wait for the result of virulence tests.

The usual dose of antitoxin for prophylaxis is 25 units per pound of body weight.

Diphtheria antitoxin has been used most frequently as a prophylactic agent in such places as orphan asylums, convalescent children homes, preventoria, etc., when a case of diphtheria appears among the inmates. The use of antitoxin has unquestionably been overdone in many such institutions. Here, again, its use should be determined by the result of nose and throat cultures and Schick tests. Only those children with a positive result in both instances should receive it. All children with a positive throat culture as well as those with the disease should, of course,

be isolated until it has been proven by virulence test or by a series of negative cultures that they are not carrying virulent organisms. The child with a positive throat culture and a negative Schick reaction should be isolated until it has been shown that he is carrying an avirulent organism or is no longer a carrier. He should not receive prophylactic antitoxin but should be inspected twice daily and have his temperature recorded at least every 8 to 12 hours. As we have pointed out elsewhere, a negative Schick reaction is not an absolute guarantee against the development of diphtheria, particularly in heavily exposed individuals.

There is nothing more important in the management of institutional children than having an accurate knowledge of the diphtherial immunity status of all inmates. An adequate program for this purpose is as follows:

If possible, all children should be actively immunized or should have had a course of active immunization started before admission. At the time of admission all children over six years of age and all children with a history of previous active immunization should be Schick tested. All those with a positive reaction should be actively immunized. All children younger than six years with no history of active immunization should be started on a course of immunization without a preliminary Schick test. All children should be Schick tested after an appropriate interval following immunization.

It is, of course, very desirable to isolate all children when admitted pending the result of a nose and throat culture. Those children with a positive culture should be kept isolated until it has been proven avirulent. Those with virulent cultures should be managed as carriers. These cultural procedures are particularly important if the child has come from an area where diphtheria is endemic or if the institution is located in such an area.

Duration of Passive Immunity. It is important to have some experimental basis for the estimation of the length of time during which passive immunity persists. There is some evidence to show that passive immunity lasts longer when the antitoxin is injected in the form of homologous serum than when heterologous serum is injected. There is considerable difference in individual animals in the speed with which antitoxin is eliminated. Studying the matter in rabbits, Glenny and Hopkins (162, 163, 164, 165) found that when antitoxin obtained from a horse is injected intravenously, there is an initial loss of 50 per cent within the first 24 hours; there is a gradual, constant loss of approximately 25 per cent more during the next week, and an accelerated loss of 50 per cent per day after the seventh or eighth day. Allowing for differences in experimental conditions and conditions in man, it is probable that one cannot count on effective persistence of passive immunization for longer than from two to three weeks.

Malignant Diphtheria. During the past few years there have been outbreaks of diphtheria of a severe, malignant form, particularly in various localized areas in central Europe, England, and Australia. In these cases the disease is most frequently localized in the nose and pharynx, less frequently involving the larynx, trachea, and bronchi. There is extensive swelling of the regional lymph glands and edema of the adjacent tissues. The cases are further characterized by generalized, severe, toxic symptoms. The mortality is very high, being 50 to 70 per cent in some series, in spite of the heroic administration of large doses of antitoxin intravenously. Much speculation has been developed to account for the etiology and pathogenesis of these very serious cases of diphtheria.

Anderson, Happold, McLeod, and Thomson (166), studying the severe diphtheria frequently seen in Leeds, England, described two distinct biological types of diphtheria bacillus which they termed the "gravis" and "mitis" forms. The difference between them was dependent upon certain biological properties which they listed as follows:

C. diphtheriae gravis	*C. diphtheriae mitis*
1. Colony like *B. xerosis*, but heavier.	1. Colony like Hoffman's bacillus, but firmer and more translucent.
2. Forty-eight hours' growth on tellurite chocolate medium; gray or gray-black.	2. Forty-eight hours' growth on chocolate tellurite medium; black.
3. Morphology: short diphtheroid, usually without granules; on Loeffler's medium, granules well marked but scanty.	3. Morphology: typical *C. diphtheriae* — usually long, with well-marked granules.
4. Pellicle and granular deposit in broth.	4. Uniform turbidity in broth.
5. Initial acid reaction in broth, usually reverting to alkaline in 2 days.	5. Acid reaction in broth persists 4–5 days.
6. Non-hemolytic.	6. Hemolytic.
7. Fermentations of *C. diphtheriae;* acid in glucose, galactose, and maltose. No acid in saccharose; *also invariably ferments dextrine, starch, and glycogen.* Fails to ferment d-glucosamine in 48 hours.	7. Recognized fermentations of *C. diphtheriae*, but inconstant results with dextrine. *Does not ferment starch or glycogen.* Ferments d-glucosamine.
8. Associated with severer cases of diphtheria; paresis common.	8. Associated with milder cases of diphtheria, paresis rare.
9. Vigorous growth on special chocolate tellurite medium.	9. Partly inhibited on chocolate tellurite medium.

In this same communication they described a number of strains as "intermediate" forms and placed cultures of the Park-Williams 8 strain

in this group. In a subsequent paper, Anderson (167) and his associates presented a more complete description of the intermediate type. They analyzed a large series of cases of diphtheria with respect to the association of the bacteriological type of organism with clinical severity of the disease. Severe cases more commonly harbored the gravis and intermediate types, whereas the mitis type was almost invariably associated with mild cases.

These authors also pointed out, and this has been confirmed many times since, that gravis strains were less virulent for the guinea pig than mitis strains. They postulated two explanations to account for this: (*a*) "The toxin is the same, but is formed more quickly by gravis in the membrane in the human throat." (*b*) "Two toxic elements, one responsible mainly for the toxic effects in animals and antigenic, the other less effective in animals than in man and possibly of the nature of an endotoxin and not antigenic." Cooper (168) and his co-workers have presented a comprehensive review of their experiences with this problem in Leeds. The reader is referred to this communication.

There appears to be general agreement that three biologically distinct types of the diphtheria bacillus exist. There is disagreement, however, with regard to the correlation of type with clinical severity of disease. A number of investigators, including Wright and Rankin (169), Menton, Cooper, Duke, and Fussell (170), and Menton (171), failed to find such correlation. Others, including Leete, McLeod, and Morrison (172) and Robinson and Marshall (173) in England, and Anderson, Goldsworthy, and Ward (174) in Australia, have found a significant association between clinical severity and bacteriological type of organism. To review further all the literature on this controversial question would be like counting votes from a ballot box. One would undoubtedly discover an expression of majority opinion, but this never provides a scientific answer to or explanation of the problem.

There is unquestioned evidence that both gravis and mitis strains produce the same antigenic type of soluble exotoxin. Both are neutralized by antitoxin produced by immunization with Park-Williams 8 toxin. This question was studied in particular by Parish, Whatley, and O'Brien (175, 176), who found that gravis strains are much weaker toxin producers *in vitro* than mitis strains. They found that mitis strains produced toxin varying from 16 to 20 L_f units in value, while with gravis strains the value varied between 0 and 6 L_f units. Complete confirmation of the identity of the exotoxins of the two strains has, of course, been provided by the innumerable routine virulence tests which have been done.

There has been some evidence presented to indicate that gravis strains may have more invasive properties than other strains. Robinson (177),

the Leeds workers, and others have studied this question. We feel, however, that sufficiently great differences have not been demonstrated to account for the pathogenesis of malignant diphtheria.

Many observers have thought that this type of severe diphtheria was due to a combined infection with the diphtheria bacillus and the hemolytic streptococcus. Indeed, in the older literature it is often referred to as septic diphtheria. Because of the assumption of a double infection, many patients have been treated by the simultaneous administration of diphtheria antitoxin and hemolytic streptococcus antitoxin or some other streptococcus antiserum. The evidence from experimental studies and from the clinical results of therapy does not justify the use of this combined form of treatment. We cannot recommend the use of any anti-streptococcus preparation in the management of malignant diphtheria at the present time.

Thus, it can be seen that many theories have been advanced to explain malignant diphtheria, none of which is adequate. We are inclined at the moment to regard it as a fulminating type of infection that may, in large part, be due to host factors in the sense of variation in susceptibility. As Schick pointed out, there would appear to be an early and rapid fixation of a lethal human dose of toxin in the tissues, and when this has occurred, even large amounts of antitoxin are of little therapeutic value. We are not without a parallel situation in other diseases, in which case, as in meningococcus infections, the same organism may be responsible for a rapidly fatal fulminating illness in one individual and a mild disease in another. In the present state of knowledge we feel that the most rational plan of management of malignant diphtheria is making a diagnosis at the earliest possible moment and administering a large dose of antitoxin intravenously as quickly as possible. In other words, our effort should be to treat the patient adequately before a lethal dose of toxin has become fixed to the tissues.

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CHAPTER XX

TETANUS (LOCKJAW) AND OTHER ANAEROBIC INFECTIONS

Tetanus

THE disease tetanus is caused by the soluble exotoxin secreted by *Clostridium tetani* during the course of its growth in a wound. This organism is a spore-bearing, anaerobic bacillus that is widely distributed in nature. Its common habitat is the intestinal tract of a large number of herbivorous animals, having been found in this location in horses, cattle, sheep, man, fowls, and various rodents. It is widely distributed in the soil in any region contaminated by human or animal excreta, particularly about barnyards and land fertilized with horse and cow manure. It has been found in virgin soil also.

There are a number of different serological types of tetanus bacilli. Tulloch (1) working during the war described four types. Since then other types have been described from time to time, bringing the total to nine at the present time. There is some difference in the geographical distribution of these types; for example, Type I is most frequently met with in America, whereas Type V is most common in China. The important consideration in this respect is that all serological types presumably secrete an antigenically similar soluble exotoxin. This is, of course, of paramount importance from the point of view of prophylaxis and treatment.

The types of wounds liable to infection are of numerous sorts; however, certain general characteristics of them are definable on the basis of the biological activity of the organism. Being an anaerobe, its growth is more apt to occur in deep wounds where oxygen is excluded. Nevertheless, circumstances may be present which favor the growth of tetanus bacilli in very superficial wounds. This is especially true when the wound is scabbed over, when small foreign bodies are present, and when infection by other organisms coexists. Tetanus has developed following such minor wounds as blisters, superficial powder burns (Fourth of July accidents), and insect bites (when the bites have occurred on the feet or legs, and dirt has been rubbed into them). Tetanus has been seen in patients in whom it has been impossible to find a lesion that was likely to harbor tetanus bacilli.

In addition, tetanus has followed a variety of other types of lesions. Tetanus neonatorum is not uncommon in certain parts of Asia and Africa due to infection of the umbilical stump. The disease has resulted from infection of the postpartum uterine contents — so-called "obstetrical tetanus" — and there have been numerous cases reported following attempts at self-induced abortion. Tetanus has also been associated with a considerable number of different surgical procedures. It has followed intestinal operations, herniorrhaphies, and particularly hemorrhoidectomies. The association with this latter type of operation is of considerable importance, since a number of studies have indicated that a fair percentage of human beings are intestinal carriers of the organism. Furthermore, instances have been reported of suture material (usually catgut) being contaminated with tetanus spores. By all odds, of course, the most common lesions associated with tetanus are those acquired at war, particularly deep puncture wounds, compound fractures, and wounds into which dirt, clothing, or other foreign bodies have been carried.

Another rather special type of tetanus which occurred during the World War was known as "cryptogenic" or delayed tetanus. In such instances the original wound healed without the patient developing tetanus. At a subsequent time when some secondary operation was done tetanus developed. In such cases tetanus spores were present in the tissues, but could not vegetate and multiply because the tissues were "healthy." At the secondary operation some of the tissues were "devitalized," and blood clots were present which made an excellent pabulum in which tetanus spores could vegetate and multiply. The passive immunity resulting from the original prophylactic injection of antitoxin was an important factor in the development of these cases. This immunity simply lasted long enough for the original wound to heal. When these facts were appreciated, army surgeons instituted the practice of giving another prophylactic injection of antitoxin at the time of secondary or reconstruction operations. Cases of "delayed" tetanus were reported as occurring as long as 200 days after the original injury.

There are certain groups among civilian populations who are more liable to tetanus than others. This is particularly true of those employed in close contact with the soil and domestic animals, such as farmers, stock raisers, stablemen, and those engaged in certain types of construction work. Individuals living in certain geographical areas are more liable to tetanus than others, particularly areas where the soil is heavily fertilized with horse and cow manure. In civilian populations there is an increased incidence of tetanus in the latter years of childhood. This is probably because of the carefree activities in which this age group engages.

A type of tetanus which merits special consideration is that which follows vaccination against smallpox. In the first place this type of tetanus is entirely preventable. In the second place cases of this sort provide unjust evidence for propaganda by anti-vaccinationists and other anti-scientific cults. This problem has received considerable attention by Francis (2), Anderson (3), Armstrong (4, 5), and McCoy and Bengtson (6) of the U. S. Public Health Service.

There is practically no evidence that such cases are due to vaccine virus contaminated with tetanus bacilli or spores. Unless shields or dressings are employed, it is practically impossible to produce tetanus in rabbits or monkeys when these animals are vaccinated with virus purposely contaminated with tetanus bacilli. Large amounts of commercial vaccine virus have been examined by Anderson without demonstrating tetanus bacilli. Furthermore, this author pointed out that from 1903 to 1913 inclusive, over 31,000,000 doses of vaccine virus were used in the United States. During this time information was obtained of only 41 authenticated cases of post-vaccination tetanus. If the virus was contaminated, it would seem that many more cases than this would have occurred. It was also pointed out that the average period from vaccination to the onset of symptoms was 20.7 days in a large series of such cases, whereas the average mortality was such as usually occurs in tetanus with an incubation period of ten days or less. From this Anderson concludes that secondary infection with tetanus bacilli probably occurs about ten days after vaccination, a time when vaccination lesions are most liable to secondary infection.

Epidemiological evidence indicates that large insertions and the use of shields and dressings predispose to post-vaccination tetanus. It is also evident that vaccinating female children on the thigh predisposes to tetanus. Thigh vaccinations are more liable to have dirt rubbed into them during the course of a child's play than arm vaccinations.

The following recommendations are indicated for the prevention of post-vaccination tetanus:

a. Strict surgical asepsis should be followed during the vaccination procedure. (Tetanus spores have been isolated from the once popular "vest-pocket" bone scarifiers.)

b. An insertion should not be more than one eighth of an inch in diameter and should be made by some method which does not remove or destroy the epidermis.

c. Shields and dressings should not be employed.

d. Female children should be vaccinated on the arm and not on the thigh.

The Factor of Infection in Tetanus. Numerous studies have been made concerning the actual factor of infection in tetanus wounds. The

various problems studied have been designed to throw light on the local conditions necessary for the development of tetanus bacilli in a wound, on the influence of other infection and the possibility of developing some immunological procedure which might enhance the destruction of tetanus bacilli or spores.

Abundant studies have shown that it is practically impossible to infect healthy living tissues even with enormous numbers of tetanus spores. In order that infection may occur, in the sense that tetanus spores vegetate and actually multiply, the tissues must be devitalized. In experimental animals various agents have been used to devitalize tissues. Tulloch (1) in his classical study employed saponin. Coleman (7) studied various tissue debilitants and found formalin the most effective. With this agent he could cause infection with as few as five tetanus spores.

In such infections the effect of antitoxic, anti-spore, and anti-bacillary sera have been studied. Tulloch found that antibacterial sera definitely stimulated phagocytosis and were type specific. The best sera were produced by the use of whole cultures rather than washed bacilli for immunization purposes. His evidence is inconclusive that such sera had any effect on experimental tetanus lesions. This problem was investigated by Coleman and Gunnison (8). They prepared anti-spore and antibacterial sera in rabbits, guinea pigs, and goats. They were unable to demonstrate that these sera contained antibodies which were effective in preventing experimental tetanus infection. They concluded that these sera did not enhance the protective value of purely antitoxic sera. It has been pointed out, of course, that even if such sera were effective, it would be practically impossible for antibodies, following parenteral injection, to get into the interior of a necrotic tissue mass where tetanus bacilli are growing.

In summary, it would seem that infection is entirely dependent upon the physical characteristics of the wound; that is, the presence of devitalized tissue, foreign bodies, or secondary infection and a reduced oxygen tension. This latter factor has been studied in considerable detail by Fildes (9). Tetanus spores will not vegetate and grow in healthy tissue, and there is no value in anti-spore or antibacterial sera. These facts suggest the necessity of adequate surgical treatment of wounds infected or suspected of being infected by tetanus bacilli.

Natural Immunity to Tetanus. There is considerable variation in species susceptibility to tetanus. It occurs spontaneously in man, horses, cattle, and sheep. It is rarely observed in dogs and goats. The ordinary laboratory animals (rabbits, mice, guinea pigs, and monkeys) are quite susceptible to tetanus. Cold-blooded animals are refractory to extremely large doses of toxin.

Whether or not man may become naturally immune to tetanus has received considerable attention in recent years; such studies being stimulated by the work of Ten Broeck and Bauer (10, 11, 12, 13). These authors, working in China, stated that tetanus is a rare disease in that country. They found that 34.7 per cent of 78 individuals carried tetanus spores in their intestinal tract. They postulated that tetanus bacilli grew and multiplied in the intestinal tract because when several such individuals were kept on practically a sterile diet for a month or more they continued to eliminate several million spores in a single stool. It was assumed that such a carrier state might result in a natural acquired immunity. These authors were able to establish a carrier state in guinea pigs and found that such animals showed considerable antitoxin in their sera and manifested an immunity to the type fed. They felt that the Type immunity was due to antibodies other than antitoxin. Similar results were obtained in human carriers. The sera of 26 individuals who were intestinal carriers contained appreciable amounts of antitoxin, whereas the sera of the majority of non-carriers were free of antitoxin. They suggested that the establishment of an intestinal carrier state might be used as a method for active immunization.

That a considerable percentage of individuals may be intestinal carriers has been found by several other investigators, among whom may be mentioned Tulloch (1), and Bauer and Meyer (14). Kerrin (15) was unable to confirm this.

The suggestion of Ten Broeck and Bauer that the carrier state may result in the development of active immunity has not been confirmed. Coleman and Meyer (16) examined the sera of 104 individuals and found agglutinins in low dilution for various types of tetanus bacilli in a considerable percentage of these. On the other hand no antitoxin was found irrespective of the presence or absence of agglutinins. Coleman (17) found it extremely difficult to establish a carrier state in guinea pigs. The serum of animals fed tetanus spores had no prophylactic value, and such animals were no more immune to injected spores than were normal controls. Coleman and Gunnison (8) found that antispore and antibacterial sera were not effective in preventing tetanus infection in guinea pigs nor did these sera enhance the value of purely antitoxic sera. Manson (18) found that the feeding of either tetanus toxin or bacilli resulted in no immunity whatever.

It would appear, then, that there is essentially no evidence that man may acquire an active immunity as a result of an intestinal carrier state. In fact, we have already pointed out that such a carrier state may be a source of some danger in connection with operations on the intestinal tract.

The Pathogenesis of Tetanus. This aspect of the problem has engaged the attention of immunologists, pharmacologists, and clinicians for many years. A clear understanding of the pathogenesis of this disease is indispensable for its intelligent treatment, particularly from an immunological point of view and in order to appreciate the limitations of serum therapy and the relative value of the various routes of administration of antitoxin. An excellent critical review of the literature on the pathogenesis of tetanus has been published by Huntington (19) of Yale University.

As early as 1892, Bruschettini (20) found tetanus toxin in the nerves but not in the tissues surrounding the point of injection and suggested that its absorption took place along the path of nerves after it had entered by way of the motor nerve endings. Since this observation, numerous studies have been made concerning the route of transport of tetanus toxin and its fixation by nervous tissue.

Marie (21) and Marie and Morax (22, 23) found that tetanus toxin could be demonstrated in the sciatic nerve 30 minutes after its injection into the hind paw of a guinea pig. If such a nerve was sectioned two hours after the toxin injection, the central stump remained free of toxin, which indicated that it had traveled centrally at a fairly rapid rate. If such a nerve was sectioned prior to inoculation, the distal stump became very toxic, whereas the central stump remained free. They concluded that toxin was absorbed through the peripheral nerve endings. They also found that when toxin was injected into the brain or spinal cord, symptoms appeared in two to four hours and after intraspinous injection no toxin was found in the peripheral nerves. The incubation period was prolonged to from 12 to 48 hours following intravenous injection. This indicated that the central nervous system was the main site of action of tetanus toxin. Roux and Borrel (24) showed that an intracerebral injection of toxin resulted in death of an actively immunized animal even though the dose was smaller than that which was necessary to cause death in a normal animal when injected subcutaneously.

The numerous experiments of Meyer and Ransom (25) confirmed and extended these observations and seemed to offer complete proof that tetanus toxin reached the central nervous system only by traveling centripetally along nerve trunks — a concept designated as "travel affinity." In addition to the facts already presented, it was shown that the progress of toxin along a nerve trunk could be blocked by an intraneural injection of antitoxin. After intravenous injection of toxin the area supplied by a blocked nerve remained free of tetanus for a long time after the disease became generalized in the rest of the body. Transection of the spinal cord after inoculation into a hind limb kept the disease localized below the level of transection.

Meyer and Ransom further concluded that tetanus toxin reached the central nervous system solely by way of the axis cylinders of *motor* nerves. If it traveled along sensory nerves, they postulated that the posterior root ganglion must act as a block to its further progress toward the cord since "tetanus dolorosus" never occurred naturally and only developed when the poison was injected into the posterior root between the cord and ganglion.

It has been shown by a number of experiments that tetanus toxin has a definite affinity for nerve tissue. Wassermann and Takaki (26) showed that a suspension of brain or cord tissue neutralized tetanus toxin much as does antitoxin. Furthermore, this union is extremely difficult to dissociate. Sedaillan and Loiseleur (27) showed that a suspension of brain tissue would flocculate with tetanus toxin. Extraction of such tissues with ether did not prevent flocculation. In spite of such striking nerve tissue affinity, tetanus toxin presumably does not destroy them. When individuals recover from tetanus, the recovery is practically always eventually complete. In this respect tetanus toxin differs from certain other neurotropic agents such as rabies virus and poliomyelitis virus which destroy nerve tissue.

Even though the evidence appeared complete that the toxin traveled along axis cylinders, this concept of "travel affinity" was challenged by a number of experiments designed to show that it might also travel along the perineural lymphatics to the central nervous system. Orr and Rows (28) demonstrated that there is an ascending lymph stream in spinal and cranial nerves, the main current of which lies in the perineural sheath. They felt that toxins and bacteria could reach the spinal cord and brain by this route. Robertson (29) supported the concept of transportation along perineural lymphatics. He showed that this was anatomically possible and that these lymphatics "side-tracked" the posterior root ganglion which accounted for the absence of such a symptom complex as "tetanus dolorosus."

Teal and Embleton (30) published a series of experiments dealing with the question of lymphatic spread of toxin. In one experiment it was shown that eight hours after inoculation of toxin into the hind foot of a rabbit, 0.1 gram of sciatic nerve and 0.1 gram of popliteal lymph node killed mice much more rapidly than 1 cc. of serum or 0.5 cc. of chyle. In another rabbit, 48 hours after inoculation, the serum and chyle were negative, whereas the sciatic nerve on the same side was still very toxic and the opposite sciatic slightly so. They point out that, since toxin is so firmly bound by nerve tissue, it is likely that the toxicity which results from the injection of such tissue into mice is probably due to the toxin in the nerve lymphatics.

These authors showed that when toxin was injected intravenously, it

quickly passed from the capillaries into the lymphatic spaces. In such an experiment the chyle secreted during the second five minutes contained several lethal doses for mice. A similar result was obtained by the intravenous inoculation of potassium ferrocyanide. However, neither the iron salt nor toxin passed the capillaries of the brain or choroid plexus, since they could not be demonstrated in the cerebrospinal fluid following intravenous inoculation. They argued that the symptoms of cephalic tetanus following intravenous inoculation of toxin were due to the spread of toxin along the cranial nerves, since they provide the shortest nerve path from the periphery to the brain.

Teale and Embleton showed that the inflammatory reaction following the injection of normal horse serum or egg albumen into a nerve trunk delayed the centripetal travel of toxin, presumably by occluding the neural lymphatics. Antitoxin injected in the same way neutralized only that toxin in the neural lymphatics. It had no effect on toxin found in either the axis cylinders or central nervous system.

Professor Abel and his associates have, in a recent series of papers (31, 32, 33, 34, 35), challenged the validity of these generally accepted theories concerning the transportation of tetanus toxin and the pathogenesis of the disease. During the course of these communications they have presented a complete and critical review of the literature on this subject. They have pointed out many errors in the experiments and the conclusions drawn therefrom which have supported the nerve transport theory for so many years. Furthermore, many instances have been cited from the literature wherein other investigators have, very rightly, challenged the validity of these theories. The reader is urged to read these papers of Abel and his associates.

It is pointed out that for this water-soluble substance, tetanus toxin, to be carried in a solid tissue structure such as axis cylinders implies a "protoplasmic streaming." There is no experimental evidence whatever that such a "streaming" occurs in axis cylinders.

It has often been stated that the incubation period, following the injection of tetanus toxin into an extremity, is longer in animals of larger size (horse, sheep) than in those of smaller size. This had been assumed to be because of the greater distance the toxin would have to travel along the longer nerves of larger animals. Abel *et al.* have pointed out that actually there is no significant variation in incubation period with the size of the animal when the dosage is accurately adjusted according to the differences in weight in different animals.

With respect to the transport of toxin to the central nervous system through perineural lymphatics Abel points out the very important fact, so completely overlooked in the past, that this is anatomically impossible. He states, "It is surprising that none of the proponents of the theory

of carriage of toxins and other drugs by way of the neural lymphatics have ever asked themselves the simple question whether their assumption of a physiologically occurring centripetal flow of lymph in nerve trunks has any foundation in fact. They seem never to have inquired whether the anatomists of our day have studied this matter and whether they have discovered at what points the lymph of nerve trunks leaves them and where it goes." He found from a review of recent anatomical literature no evidence that neural lymphatic vessels enter the spinal canal at any intervertebral junction. The lymphatics of the upper sciatic nerves, for example, drain into deeply lying inguinal glands. There is no anatomical evidence that any substance can be carried into the central nervous system by perineural lymphatics.

Localized tetanus has caused much speculation among students of tetanus and has been used by the proponents of the neural transport theory and its variants as a supporting argument. In the experimental animal the first symptom of tetanus generally is a local spasm of the muscles of the extremity into which the toxin was injected. Furthermore, numerous instances have been described of various types of local tetanus in man. Such human cases have been described in considerable detail by Courtois-Suffit and Giroux (36).

Abel, Hampil, and Jonas in a series of very carefully performed experiments have shown that experimental local tetanus is due to the direct action of the toxin on the muscles and not on nerves. Dogs were employed in their experiments because of the larger size of their nerves. Very carefully quantitated doses of toxin in small volume were used. When the sciatic nerve was carefully isolated, kept free from contact with muscles by packing with wet gauze, and leakage guarded against, local tetanus did not follow an intraneural injection of a small dose of toxin. On the other hand, as little as $\frac{1}{200}$ the intraneural dose of toxin would cause severe local tetanus when injected directly into the muscles.

These authors conclude, both from a critical review of the literature and from their own experimental work, that tetanus toxin is carried to the central nervous system and throughout the body by the blood stream only. They further point out that it is very probable that tetanus toxin also has a direct action on muscles or myoneural junctions, as well as a specific action on the central nervous system. This, they argue, would better explain the symptoms in the natural disease. For example, deep narcosis or anesthesia will relieve a patient of generalized convulsions, which are undoubtedly of central origin, but will not completely relieve the severe, continuous muscle spasms.

The thesis of Abel *et al.* that tetanus toxin is carried from the site of infection only by lymphatics and the blood stream, and that it is trans-

ported to the central nervous system by the blood stream, would appear to have much evidence to support it.

Standardization of Tetanus Antitoxin. The technical procedures employed in the standardization of tetanus toxin and antitoxin are similar to those used for diphtheria toxin and antitoxin. In the American method of standardization, based upon the work of Rosenau and Anderson (37), the unit of tetanus antitoxin is defined as ten times the least amount of serum necessary to save the life of a 350-gram guinea pig for 96 hours against the official test dose of standard toxin. The test dose consists of 100 minimal lethal doses (the M.L.D. being defined as the least quantity of toxin which, when injected subcutaneously, will kill a guinea pig weighing 350 grams within 96 hours) of a precipitated toxin preserved under special conditions at the National Institute of Health, Washington. A standard antitoxin is likewise preserved at the National Institute of Health.

Unfortunately other methods of standardization have been employed in other countries, the German, French, and American units being quite different. Comparative studies made under the auspices of the Health Organization of the League of Nations indicate that 1 German unit is equivalent to 66 American units and to 3750 French units. The Permanent Committee on Standardization (38) recommended that an International unit should be established equivalent to one half the American unit and that the standard antitoxin should be kept at the Serum Institute in Copenhagen.

Prophylaxis by Passive Immunization. While there is justifiable doubt as to the therapeutic value of tetanus antitoxin, there can be no doubt whatever as to its prophylactic value. Its value for this purpose has been thoroughly proven by the experiences during the World War, as well as by numerous observations on civilian populations. A few studies may be cited to show this.

TABLE I

THE REDUCTION IN THE INCIDENCE OF TETANUS FOLLOWING FOURTH OF JULY INJURIES AFTER THE INTRODUCTION OF PROPHYLACTIC ANTITOXIN

YEAR	TOTAL INJURED	TETANUS CASES	PER CENT OF TETANUS CASES	DEATHS FROM TETANUS	TETANUS MORTALITY PER CENT
1903	4,449	415	9.35	406	97.8
1904	4,169	111	2.52	87	78.4
1905	5,176	104	2.01	87	84
1906	5,466	89	1.45	75	84
1907	4,413	73	1.65	62	84
1908	5,623	76	1.35	55	72.4
1909	5,307	150	2.82	125	82.3
1910	2,923	72	2.46	67	93
1911	1,603	18	1.12	10	55.5
1912	988	7	0.71	6	86
1913	1,163	4	0.35	3	75
1914	1,506	3	0.2	3	100

Taken from Robertson (39).

Robertson (39) in a review of the prophylactic use of antitoxin cited the experiences in connection with Fourth of July injuries. It had been observed that roughly 10 per cent of such injuries were followed by tetanus which was usually very virulent. In 1903 the American Medical Association started a campaign to relieve this dreadful situation. Among other things the use of antitoxin prophylactically was urged and thoroughly publicized among the medical profession. As is indicated in the table on the opposite page taken from Robertson's paper, the effect was obvious the year following the inception of this campaign.

The figures for the British Army during the World War, as analyzed by Bruce (40), present striking evidence of the prophylactic value of tetanus antitoxin in preventing the disease. Furthermore, prophylactic antitoxin seemed definitely to have modified the course of the disease in several respects in those cases developing tetanus even though having been given prophylactic treatment. The incubation period was generally prolonged. Moreover, there was a considerable reduction in mortality in protected cases as is shown in Table II.

TABLE II

COMPARISON OF THE RATE OF MORTALITY IN THE PROTECTED AND UNPROTECTED

	NUMBER OF CASES	RECOVERED	DIED	RATE OF MORTALITY PER CENT
Protected	889	676	203	22.5
Unprotected and unrecorded .	559	260	298	53.3

Taken from Bruce (40).

The use of prophylactic antitoxin seemed to have some effect on the increased incidence of local tetanus as the war progressed. It is important to note also that the mortality is very low in local tetanus.

It is unfortunate that no specific rules can be given in regard to which wounds constitute a definite tetanus risk. There is no practical bacteriological procedure that is reliable in helping to arrive at this decision. As we have pointed out elsewhere, tetanus has followed seemingly insignificant wounds and in some cases no demonstrable wound. It is quite obvious that antitoxin cannot be given following the frequent minor cuts and scratches we all receive. There are many instances where the physician is quite justifiably in a quandary as to whether antitoxin should be given. On the other hand, there are certain injuries concerning which there can be no doubt at all. There are certain general principles which should guide one in making these decisions, many of which have

already been alluded to in our discussion of the factor of infection in tetanus.

Antitoxin should always be given following such wounds as compound fractures, gunshot and penetrating wounds, deep cuts, and puncture wounds, especially when dirt, clothing, splinters, and other foreign bodies are apt to have been carried into them. It should be administered following much less serious wounds if they are likely to contain dirt contaminated with horse manure. A careful history should always be taken regarding the circumstances under which a wound has occurred. With respect to the numerous types of minor wounds the physician's judgment will have to decide whether to give antitoxin. Any wound that is apt to contain dirt or foreign bodies is liable to be followed by tetanus.

The dose of antitoxin varies from 500 to 3000 American units, depending on the character of the wound. Larger doses should be employed in the more severe injuries such as compound fractures.

It should further be borne in mind that antitoxin administered in this manner affords passive immunization only. Such antitoxin is gradually eliminated from the body, being almost entirely eliminated by the end of two weeks. For this reason a second dose of antitoxin should be given a week after the first in cases of bad wounds which do not heal properly or continue to have considerable secondary infection. As has been pointed out elsewhere, antitoxin does not rid a wound of tetanus bacilli.

We have already indicated that tetanus may follow secondary operations such as removing bone sequestra, etc. In these instances the original injury is usually very severe. Under such circumstances it is advisable to administer another dose of antitoxin before such secondary operations. Bruce reported 102 cases of tetanus following such secondary operations.

Obviously, in all instances where antitoxin is administered, particularly second doses, due precautions should be taken against the possibility of serum reactions. In this connection also, bovine antitoxin is of value. An individual who is markedly hypersensitive to horse antitoxin may tolerate an equivalent amount of bovine antitoxin without reaction. Prophylactic antitoxin should be given intramuscularly or subcutaneously as soon as possible after the injury. According to the figures of Bruce, it is equally effective when administered on the day of the injury, or either of the first two days after the injury. The proper surgical care of dirty wounds is, of course, of the utmost importance.

Prophylaxis by Active Immunization. Tetanus toxoid has been developed and, very probably, will prove to be a useful product for active immunization against tetanus, particularly in those individuals who, by occupation, are especial tetanus risks. Soon after Ramon showed that diphtheria toxin could be rendered nontoxic by treatment with formal-

dehyde, Descombey (41) found that the method worked equally well with tetanus toxin. This changed toxin or toxoid (anatoxin) retained its antigenic properties in spite of the loss of toxicity.

Tetanus toxoid is prepared by the addition of 0.3 to 0.4 per cent formaldehyde to tetanus toxin and incubating the mixture at 37° C. for several weeks. The end point of the period of incubation is determined by the loss of toxicity of the material for laboratory animals. By such treatment a toxin with an M.L.D. of 0.001 to 0.0001 cc. can be so reduced in toxicity that 10 cc. will fail to kill a guinea pig. Such toxins are considered to be adequately changed to toxoid when 5 to 10 cc. fail to cause symptoms of tetanus when injected into a guinea pig. Bergey (42) and others have shown that the best toxoid is derived from the most potent original toxin.

A number of papers have appeared concerning the production of antitoxin in human beings and animals following two or three injections of toxoid at biweekly or triweekly intervals. Such studies have been published by Ramon and Zoeller (43, 44), Sacquépée (45), Lincoln and Greenwald (46), Sneath (47, 48, 49), and Bergey and Etris (50, 51, 52). Ramon (53) has published a review of this subject. In general, these studies have shown that the rise in antitoxin following the primary stimulus is rather slow, reaching a maximum in from three to five months. After this interval of time, in persons receiving two or three injections of toxoid, the antitoxin level in the serum averages about that occasioned by the injection of the usual prophylactic dose of antitoxin. However, in a large group of immunized individuals, there will be considerable variation in the level of antitoxin in their serum. There are occasional individuals in whom a very poor antitoxin response follows such injections of toxoid.

This active immunity apparently lasts for a considerable period of time. The serum antitoxin level seems to maintain itself for 12 to 18 months and then begins to fall slowly. Ramon and Zoeller injected a large group of individuals with tetanus toxoid, and in some of them the serum antitoxin level following this varied from 0.5 to 1 unit per cc. Four years later 12 of these individuals had serum antitoxin contents of from $\frac{1}{500}$ to $\frac{1}{6}$ unit per cc.

It has also been shown that a secondary stimulus (an injection of toxoid in a person immunized some time previously) is followed by a very rapid rise in antitoxin to a new high level. In from 7 to 15 days, such a secondary stimulus is followed by a rise in antitoxin which may be several hundred times greater than the antitoxin level just prior to the secondary stimulus. The level attained after such a stimulus usually is many times higher than the optimum level following the primary stimulus. The importance of this will be discussed later.

Bergey has shown that when tetanus toxoid is precipitated with 2 per

cent alum and washed according to the method of Wells, Graham, and Havens (54), it is not followed usually by the annoying reactions which sometimes follow the use of non-precipitated toxoid. Because alum-precipitated toxoid is retained longer in the tissues, it acts as an antigenic stimulus for a longer time than regular toxoid. Bergey found that a single dose of alum toxoid is often followed by an antitoxin content similar to that following three injections of regular toxoid. Jones and Moss (55) report similar findings following the injection of alum-precipitated tetanus toxoid.

There is no question but that tetanus toxoid is an active immunizing agent and that it would seem to be perfectly possible to immunize individuals against tetanus. However, it remains to be seen to what extent it will receive practical application. The incidence of the disease in the general population is sufficiently low to make it questionable whether large-scale immunization would be advisable. Unquestionably, such immunization should be of great value in that portion of the population whose occupations make them special tetanus risks, *i.e.*, agriculturists and those engaged in stock raising and military campaigns.

In actively immunized individuals, even though the immunizing injections were given a considerable time previously, and their serum antitoxin may be very low at the moment, it should be possible to protect them against tetanus by administering another dose of toxoid at the time of an injury. Thus, when a previously immunized individual is injured, he is given an injection of tetanus toxoid instead of tetanus antitoxin. Such a procedure, of course, avoids the development of serum sensitivity. There will then be a sufficient rise in his own antitoxin level during the next few days to protect him against tetanus. Furthermore, it is possible, in soldiers and others running a particular tetanus risk, to keep their serum antitoxin at an effective level at all times by giving a single injection of toxoid every 12 to 18 months after they have been immunized.

It should be pointed out and emphasized that tetanus toxoid is of no value as a therapeutic agent. It should never be given during the course of the disease. It is solely an active immunizing agent and should be used only for prophylaxis against the possible development of tetanus following a future injury. Furthermore, because of the slowness of antitoxin development following a primary stimulus, it should never be used as a prophylactic agent at the time of an injury in a person who was not actively immunized previously.

It is regrettable that there is no simple test, analogous to the Schick and Dick tests, to determine susceptibility to tetanus. An estimation of the degree of protection in an individual can be determined only by measuring the amount of antitoxin in his serum. This is an elaborate

procedure which would not be practical in any large group of individuals. Without such a measurement it is impossible to know if an individual is adequately protected by active immunization. This difficulty may prove to be a stumbling block in the way of large-scale immunization against tetanus. Uncertainty cannot be tolerated when one is taking care of an individual with a serious injury that is likely to be infected with tetanus bacilli.

Tetanus toxoid is usually administered in three 1.0 cc. doses injected subcutaneously at various intervals of time. Sneath employed three doses of 1.0, 1.5, and 1.5 cc. With alum-precipitated toxoid, two doses, with a considerable interval between, would seem to be adequate. A third dose is given at the time of a subsequent injury. It is important to emphasize that the longer intervals of time between injections, preferably three or four weeks, are desirable.

Specific Treatment of Tetanus. There are three phases which should be followed in the treatment of patients with tetanus:

1. Proper surgical care, *i.e.*, débridement, removal of foreign bodies, etc., from the infected wound. Cauterization of such wounds should never be done. The necrotic tissue resulting from such procedures provides an excellent medium for the growth of tetanus bacilli.
2. Detailed and well-planned symptomatic treatment with special reference to properly selected sedatives administered in effective dosage.
3. Specific treatment with tetanus antitoxin.

We are concerned here only with a discussion of this latter phase of treatment.

Antitoxin is of limited, if not of doubtful, value in the treatment of tetanus. As we have already pointed out, tetanus toxin is so firmly bound by tissue cells that it cannot be dislodged or neutralized by antitoxin. On this basis, then, one should not expect the administration of antitoxin necessarily to relieve a patient of tetanus symptoms, which symptoms, of course, are due to that toxin bound by tissue cells. This reasoning, in fact, has been amply supported by clinical observation.

A review of mortality statistics does not impress one regarding the value of antitoxin. Bruce, in analyzing 1458 cases which occurred in home military hospitals during the course of the World War, reports a mortality rate of 34.8 per cent for 1437 cases. However, the majority of these cases received prophylactic antitoxin, and we have already pointed out the effect of this on mortality. In this same series the mortality was 53.3 per cent in 559 cases who presumably received no prophylactic antitoxin. Golla (56) made a statistical analysis of the cases occurring during the war. He found that there was essentially no difference in the mortality as compared with cases in pre-serum days.

More recent figures reported from civilian hospitals are not more

encouraging. In 1924, Graffagnino and Davidson (57) reported on 596 cases from the New Orleans Charity Hospital with a mortality of 67.4 per cent. Two additional series have been reported from the same institution. In 1930, Graves (58) reported 217 further cases with a 52 per cent mortality, and in 1935 Boyce and McFetridge (59) reported 185 additional cases with a mortality of 59.5 per cent. Calvin and Goldberg (60) have reported 183 cases from the Cook County Hospital in Chicago with no appreciable change in mortality over a 15-year period. Miller and Rogers (61) reported a 47 per cent mortality in 33 cases treated at the Massachusetts General Hospital in Boston.

In our opinion any benefit that might be derived from antitoxin in the treatment of tetanus would be, in a sense, prophylactic. That is, its benefit is probably limited largely to neutralization of uncombined toxin circulating in the blood stream and lymphatics and possibly that which is continuing to be absorbed from the lesion. For this reason, of course, its use as a therapeutic agent is definitely indicated.

Two problems in the serum treatment of tetanus, namely, the route of administration and the dosage of serum, have received considerable discussion.

We feel that the intrathecal administration of antitoxin, which was so popular a few years ago, is to be condemned. In the first place there is no logical reason for it, and in the second place it would seem to be definitely harmful. If the latest evidence, as reviewed above, concerning the transportation of toxin by the blood stream is correct, there should be no reason for intrathecal administration. Even if the older theories are correct, we see no reason for using this route. To reach the motor cells and their area in the anterior horns, antitoxin in the subarachnoid space would have to pass the pial membrane and be absorbed across a considerable space of tissue. Actually, such serum is absorbed relatively quickly by the blood stream and would be carried to the motor cells by this mechanism.

Furthermore, any substance — foreign serum, human serum, air, or even physiologic salt solution — introduced into the subarachnoid space is a definite irritant and causes considerable inflammatory reaction. The patient with severe tetanus already has a very hyperexcitable central nervous system, and this additional irritation is definitely harmful. We have seen several patients go into collapse and die within a few hours of the intraspinous administration of antitoxin. Klemmer and Crosland (62) have reported 96 cases of tetanus with a 57 per cent mortality. Of these cases, 19 received serum intraspinally, as well as by other routes. The mortality in this group was 68 per cent.

In recent years there has been a growing tendency to employ very large doses of serum, *i.e.*, 300,000 to 500,000 units. We fail to see any

rational for this. With respect to benefit claimed for large doses, Boyce and McFetridge succinctly summarize the opinion of many authorities by stating that "some patients died too promptly to receive the amount they needed, while others lived long enough to get more than they needed."

In our opinion a maximum total dosage of serum should be not more than 100,000 units. We feel that one should be guided as to dosage more by the character of the infected wound, together perhaps with the length of the incubation period, than by the gravity or character of the symptoms presented. Patients with serious wounds that are apt to be heavily infected with tetanus bacilli should receive the larger doses of serum. We do not advise the continuation of serum for longer than three or four days. In milder cases considerably smaller doses of serum than the above are probably adequate.

Gas Gangrene

While many of the organisms associated with gas gangrene had been recognized for many years, it remained until the World War for their pathogenic significance to be clearly described. Largely as the result of the work of French (63) and English (64) investigators it became apparent that this group of anaerobic organisms was the cause of those severe lesions following trauma described as gas gangrene. The development of methods of management of these serious wound infections is a classical example of fruitful co-operative effort between bacteriologists and surgeons. It became apparent during the war that gas gangrene was generally a mixed infection, several organisms of this group being associated together. Monobacterial infections were very uncommon.

The gas gangrene organisms are, generally speaking, saprophytic bacteria. Their normal habitat is the intestinal tract of animals and decaying animal and vegetable matter. Resistant spores are formed as a result of which they survive for long periods in soil and become widely disseminated in dust. It is only natural, therefore, that severe traumatic wounds should be contaminated by organisms of this group.

Certain of these anaerobes, notably *Cl. welchii*, *C. septique*, and *C. oedematiens*, are acutely pathogenic. They not only invade the local tissues in the region of the wound but, on occasion, may invade the blood stream and other organs. Such organisms as *Cl. fallax* and *C. histolyticum* are less pathogenic than those mentioned above. *Cl. histolyticum* has the remarkable property of digesting living tissue. Other anaerobes, such as *Cl. sporogens* and *Cl. bifermentans*, have essentially no pathogenic property as individuals. Nevertheless, they may produce harmful effects in association with other anaerobes. The production of a toxin of varying degrees of potency is a characteristic of all the gas gangrene anaerobes.

In this discussion we shall briefly consider only the more important of these microorganisms.

Cl. welchii. This organism was discovered in 1892 by Welch and Nuttall (65) and was called by them *B. aerogenes capsulatus.* It was also known for a time as *B. perfringens. Cl. welchii* occurs more frequently in gas gangrene than any of the other anaerobes. Moreover, it is of great economic importance in the agricultural industry, being the cause of several important animal diseases, among which may be mentioned lamb dysentery.

The soluble toxin of this organism was first obtained in a definitely recognizable form by Bull and Pritchett (66, 67) in 1917. The production of the toxin in their cultures depended upon the presence of fresh muscle tissue and glucose in the broth in which the organism was grown. They found no variation in the ability of the different strains of *Cl. welchii,* irrespective of the source, to produce the toxin, but toxin production seemed to be directly proportional to virulence. Hartley (68) secured good toxin by growth in a t yptic digest broth with chopped meat to a depth of about one inch in the bottom of the culture flask.

The Welch bacillus apparently produces at least four different types of soluble exotoxin. A single strain may produce all four types. Some of these types are responsible for certain diseases in animals. For example, the B toxin is responsible for the production of lamb dysentery and the C toxin is the cause of an enteritis of sheep known as "struck." This organism also produces a potent hemolysin.

Antitoxin can be produced in horses by the intramuscular injection of toxin, commencing with small amounts and gradually increasing the dosage. Weinberg and Prévot (69, 70) produced satisfactory antitoxin by immunization with toxoid. These authors also used a so-called "anaculture" for immunization, which consisted of a mixture of organisms and toxin which had been treated with formalin for 8 to 15 days.

Hartley (71) has reported on an international standard unit which was accepted by the Health Organization of the League of Nations. This unit is the specific antitoxic activity contained in 0.322 mg. of a dry-stable specimen of antitoxin prepared by the National Institute of Health in Washington. Titrations, using toxin-antitoxin mixtures, are usually carried out by intravenous injection of mice.

***Cl. Septique* (*Vibrion Septique, B. Oedematiens Maligni*).** This organism was isolated by Pasteur and Joubert (72) in 1877 and named by them *Vibrion septique,* a name commonly given to it at the present time. This organism, according to Weinberg and Séguin, occurred in about 12 per cent of the wounds examined by them. It is usually associated with other anaerobes, particularly *Cl. welchii.*

Toxin can be prepared by methods similar to those used for the Welch

bacillus. Likewise, potent neutralizing antitoxin can be produced by immunization of horses.

The proposed international standard for Vibrion septique antitoxin submitted by Hartley and White (73) and recommended by the Committee on Standardization of the League of Nations is, "That the dry stable preparation of gas gangrene antitoxin (Vibrion septique) prepared at the National Institute for Medical Research, London, from material supplied by Dr. Weinberg, of the Pasteur Institute, Paris, be accepted as the International Standard for this antitoxin and that the specific antitoxic activity contained in 0.2377 mg. of the dry standard preparation be defined as one international unit."

This unit of antitoxin neutralized about 24 lethal doses for the mouse of the particular toxin used in the experiments concerned with the study of this standard.

Two methods were described for assaying antitoxin in test animals. In the first, mixtures of toxin and antitoxin in amounts of 0.5 cc. were injected into the caudal veins of mice. In the second method, toxin-antitoxin mixtures in amounts of 0.2 cc. were injected intradermally in light-colored guinea pigs; the reactions being read after 24 and 48 hours.

***Cl. Oedematiens* (*B. Novyi*).** This organism was first described by Novy (74) in 1894. It was rediscovered during the war by Weinberg and Séguin, who named it *B. oedematiens*. The latter investigators isolated this organism in 34 per cent of the war wounds examined. *Cl. oedematiens* produces a toxin of great potency.

Both toxin and antitoxin are produced by methods similar to those employed for the other anaerobes in this group. The International Standard unit of antitoxin recommended by the League of Nations Commission (75) is, "That the dry stable standard for gas gangrene antitoxin (*oedematiens*) prepared at the Statens Serum Institute, Copenhagen, be accepted as the International Standard for this antitoxin and that the specific antitoxic activity contained in 0.2681 mg. of the dry standard preparation be defined as one international unit."

The method recommended for the assay of antitoxin consists of the injection of mice with toxin-antitoxin mixtures in 0.2 cc. quantities. The mixtures are permitted to stand at room temperature for one hour prior to injection. The animals are observed for three days and the number of deaths recorded.

***Cl. Histolyticum*.** This organism was isolated from wounds by Weinberg and Séguin (76, 77). It has the unique property, when injected intramuscularly, of digesting living tissue. In an experimental animal the tissue may be so destroyed as to leave the bone denuded. This organism produces a true, antigenic exotoxin.

Cl. histolyticum antitoxin has been produced by methods similar to those in use for other anaerobic organisms. For standardization the League of Nations Commission (78, 79) recommends, "That the dry stable preparation of B. histolyticum antitoxin established by the State Serum Institute of Copenhagen be adopted as the international standard of that antitoxin, and that one international unit be defined as the specific antitoxic activity contained in 0.3575 mg. of the said dry standard preparation." Both the mouse intravenous method and guinea pig intradermal method gave reliable results.

Antibacterial Antibody in Gas Gangrene Infections. During recent years studies have been made concerning the antigenic composition of organisms associated with gas gangrene. Such studies have been extended to include experimental observations on antibacterial serum. Felix and Robertson (80) showed that some of these anaerobes contained the "O" and "H" antigens. They found that only the O antibody was active in complement fixation. Robertson and Felix (81) showed that a serum containing only O antibody was effective both prophylactically and therapeutically in animals experimentally infected by injection of homologous spores (Vibrion septique) together with calcium chloride as an activator. These observations were confirmed and extended by Henderson (82, 83). These studies suggest that a serum containing O antibody as well as antitoxin would be desirable. The O antibody is type specific, however, and with some of these organisms, at least, a polyvalent antibacterial serum would be necessary.

The Prophylactic and Therapeutic Use of Gas Gangrene Sera. Because so-called gas gangrene lesions, particularly those associated with severe trauma, are usually due to mixed infections, it is advisable to use a polyvalent serum, particularly one containing antitoxin, for the four organisms we have described above. Obviously, of course, the possibility of tetanus should be given proper consideration. A monovalent serum may be used in special cases where a monobacterial infection has been proven. Moreover, treatment may be started with a mixed serum and changed later to correspond to the bacteria flora present.

The usual therapeutic doses of serum recommended have been 80 to 100 cc. given subcutaneously and intramuscularly in the region of the wound and intravenously. Larger doses have been given in particular cases. Smaller doses (10 to 30 cc.) have been used prophylactically. It should be realized that we do not have sufficient data upon which to recommend definite dosage. Furthermore, circumstances attendant upon each individual case must be taken into account by the physician in deciding upon dosage. Such factors as the extent of the trauma, the amount of contamination with dirt, the amount of general toxemia, and the time elapsing between the occurrence of the injury and the institution

of treatment are important. Of great importance also is the proper surgical management of such cases.

It is difficult to arrive at an accurate decision as to the value of serum in the prophylaxis and treatment of gas gangrene. It was not until toward the close of the World War that proper sera were developed. As a result only a limited experience of their use occurred before the Armistice. Much of our information is dependent upon results obtained in civil hospital practice since that time. Such results are, naturally, difficult to appraise and control because of the many variables concerned in each individual case. It would appear, however, from a number of reports (84, 85, 86, 87, 88, 89) that the mortality from these infections has been definitely reduced by the use of serum. This, together with the experimental results in animals, justifies the continued use of serum in the treatment of gas gangrene infections.

Botulism

Cl. botulinum was described by van Ermengem (90) in 1897. He observed an outbreak of food-poisoning involving 34 persons who had eaten a particular salted ham. He isolated a sporulating anaerobe from the ham and from the spleen of one of the fatal cases and showed that living cultures and the soluble filtrate from such cultures produced in experimental animals all the symptoms observed in the human cases. These observations were soon confirmed by Römer (91). Since then this organism has been shown to be the cause of numerous outbreaks of botulism and has also been shown to cause several animal diseases.

Cl. botulinum is a strict saprophyte, causing illness by the very potent exotoxin which it secretes during its growth in meat and in canned foods in particular, where, owing to the manner of preparation and canning ideal cultural conditions are present for a resistant spore-forming anaerobe. The toxin is unusual in that it is not readily destroyed by the digestive juices. Intoxication follows the ingestion of food containing the toxin.

Cl. botulinum forms two distinct toxins, types A and B, which are commonly associated with human botulism. These toxins are antigenically distinct in that their respective antitoxins will not cross-neutralize. In addition, three other toxins, C, D, and E, have been described and are associated with certain animal diseases.

Kempner (92), in 1897, showed that the toxin of this organism caused the development of a powerful, specific antitoxin in goats. Potent antitoxin can be produced in horses or mules by immunization with toxin-antitoxin mixtures or with toxoid. Serum for standardization is provided by the National Institute of Health (93, 94, 95). The standard antitoxin for types A and B was originally based on 10 times the amount

of antitoxin protecting against 100 M.L.D. of the toxin being used. A satisfactory serum should contain 200 units of antitoxin or more per cubic centimeter.

Treatment of Botulism with Antitoxin. Antitoxin is the only known remedy that is likely to be of benefit in the treatment of this disease. It should be administered intravenously in doses of 20 to 40 cc. early in the course of the illness. It is unlikely to be of benefit when neurological symptoms are present and in an advanced stage. It should be possible, however, to make an early diagnosis in some cases when a number of individuals are involved in a particular outbreak.

Antitoxin, in a dose of 10 cc. intramuscularly, should be used prophylactically in persons who have consumed the same food suspected or known to have caused an outbreak of botulism.

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CHAPTER XXI

SCARLET FEVER

SCARLET FEVER is an acute infectious disease, principally of childhood, with an incubation period of two to seven days. It is characterized by a rather sudden onset of fever, malaise, and vomiting, with a characteristic erythematous rash appearing early in the febrile course and lasting a variable period of time. There is a typical involvement of the throat and tongue — the so-called "scarlatinal angina." A widespread desquamation of the epidermis usually occurs about two weeks after the onset.

There are many analogies between scarlet fever and diphtheria with one important exception. In scarlet fever one is dealing with at least two important disease-producing factors. An important part of the clinical picture is due to the soluble exotoxin of the hemolytic streptococcus. Indeed, those features which characterize the disease as an entity, such as the rash, are due to this toxin. In addition, many of the features, principally the various complications involving the upper respiratory tract, are septic in their origin, being due to actual invasion by the streptococcus. It is important to keep in mind a distinction between the toxic and septic features of the disease in a consideration of such procedures as active immunization and the therapeutic use of specific antitoxin.

An interesting feature of scarlet fever which likewise has an important bearing on prophylaxis and treatment is the great variability in the severity of the disease, not only in different localities in the same season but in the same locality in different seasons. Throughout the past there appears to have been periodic waves of severe scarlet fever. At the present time the disease is very mild in America and England, with a mortality rate of about 1 per cent. On the other hand, certain regions in the Balkan States are experiencing a severe toxic form of the disease with a high mortality. In certain provinces of China a very malignant septic form has been reported during the past few years.

Etiology. The development of knowledge of the etiology of scarlet fever was extremely slow, and in view of our present information many of the older observations are of unusual interest. It had long been noted that long-chain streptococci were associated with the angina of the disease. Dochez (1) stated that Klein, in 1885, isolated a streptococcus

from ulcerative lesions in the udders of cows belonging to a farm in Hendon, England. Milk from the infected cows was consumed by persons who subsequently developed scarlet fever. In 1900, Baginsky and Sommerfeld (2) found such organisms in each of 700 cases. Following the demonstration by Schottmüller (3), in 1903, of the difference between streptococcus viridans and the hemolytic streptococcus on blood agar plates, it became evident that the hemolytic variety was constantly associated with the disease and its complications. Hektoen (4), in 1903, isolated the streptococcus by blood culture in 12 per cent of his cases. He observed that this evidence of bacteriemia was not in itself a bad prognostic sign.

Realizing that the hemolytic streptococcus was causally related to so many septic processes, it was only natural that attempts were made to associate a particular variety of the organism with such a specific clinical entity as scarlet fever. Moser and von Pirquet (5) and Meyer (6), in 1902, and Rossiwall and Schick (7), in 1905, examined scarlatinal strains by agglutination and thought they could differentiate them from organisms isolated from other sources. These findings were not confirmed by Aronson (8), Hasenknopf and Salge (9), and Neufeld (10). In view of the technical difficulties in typing hemolytic streptococci, unknown at the time of these investigations, it is not surprising that such conflicting evidence was obtained. Later, we shall deal with the grouping of scarlatinal strains at greater length, since it is so important in considering the epidemiology of this and other streptococcus diseases.

The observations of Gabritschewsky (11) in 1907 are of unusual interest. This investigator made a vaccine with killed cultures of hemolytic streptococci isolated from cases of scarlet fever with which he hoped to immunize against the disease. He noted a number of reactions following the injection of the vaccine. Some individuals developed a marked local erythematous reaction, while others developed a general reaction including fever, malaise, and a transient erythema as well as injection of the fauces and tongue in some. The latter reaction clearly resembled the so-called "strawberry tongue" of scarlet fever. He further noted that individuals who recently had an attack of the disease did not develop such reactions after the injection of the vaccine. Some time before, Moser (12) had prepared an antiserum by immunizing horses with streptococci of scarlatinal origin. Excellent therapeutic results were reported following the use of this serum. Gabritschewsky found that the reactions to his vaccine could be prevented by the previous administration of Moser's serum. Reasoning in retrospect it is possible that Gabritschewsky was observing susceptibles and non-susceptibles to scarlet fever on the basis of the reaction to his vaccine; soluble scarlatinal exotoxin was present in his vaccine; and Moser's anti-

scarlatinal serum contained scarlatinal antitoxin capable of neutralizing the toxin. Unfortunately, these observations were not developed to their logical conclusion for several reasons. Others failed to confirm Moser's therapeutic claims, and the use of antiserum was given up. There was a growing supposition that a filtrable virus was the primary etiological agent and that the hemolytic streptococcus was a secondary invader. The disease could not be reproduced in all its manifestations in experimental animals, although some partially successful experiments such as those of Landsteiner, Levaditi, and Prasek (13) were reported.

In 1914, Krumwiede, Nicoll, and Pratt (14) described an instance in which a laboratory worker accidentally sucked a suspension of living hemolytic streptococci into her mouth. She developed and passed through a typical attack of scarlet fever after an incubation period of three days. Though accidental, this was definitely an instance of the successful transmission of the disease.

Dick and Dick (15), in 1921, attempted to reproduce the disease by swabbing the throats of a group of volunteers with various organisms, including hemolytic streptococci, isolated from the throats of scarlet fever patients. None developed the disease although a few developed acute tonsillitis. In 1923 these investigators (16) carried out an additional series of experiments in which the transmission of the disease was successful. The throats of five volunteers were swabbed with a culture of hemolytic streptococcus isolated from the septic finger of a nurse ill with surgical scarlatina. Three of the volunteers showed no reaction, one developed acute tonsillitis, and one passed through a typical attack of scarlet fever. As we shall see later, it is quite significant that one of these individuals developed an acute tonsillitis without the usual manifestations of scarlet fever.

Finally, the mechanism of production of the important symptoms of scarlet fever was made clear by the investigations of Dick and Dick (17, 18) of the toxic properties of filtrates of broth cultures of scarlatinal strains of hemolytic streptococci. They showed that an intradermal injection of 0.1 cc. of a 1 : 1000 dilution of such a filtrate gave rise to an area of circumscribed erythema in a certain proportion of individuals who never had scarlet fever. This reaction became the Dick test as we now know it. It soon became evident that the injection of a larger amount of this material occasionally gave rise to general symptoms of scarlet fever. The Dicks (19) immunized horses with such filtrates and produced an antitoxin capable of neutralizing the toxic action of their filtrates. Dochez (20, 21), who was occupied with the problem at about the same time and suspected a specific toxin, immunized horses with living scarlet fever streptococci injected subcutaneously, together with agar, in order that they might produce all their metabolic products within

the horse. Blake, Trask, and Lynch (22) showed that Dochez's serum would induce a positive rash extinction test and that a marked beneficial effect followed its therapeutic use in early cases of scarlet fever.

In spite of this chain of positive evidence to incriminate the hemolytic streptococcus the opinion is still occasionally expressed that the primary agent in scarlet fever is a filtrable virus. There is no positive evidence whatever to support this view. Kinloch, Smith, and Taylor (23) did a crucial experiment in this regard, based upon the fact that Dick toxin is relatively heat stable. A toxic filtrate was heated to 55° C. for one hour, after which a large dose was injected into a volunteer who developed a typical rash and symptoms of scarlet fever, followed in due course of time by desquamation. It was pointed out that a filtrable virus would very likely be inactivated by such heating.

We may summarize the facts which convince us that the hemolytic streptococcus is the sole etiological agent in scarlet fever as follows: (*a*) Hemolytic streptococci are always present in the throats of scarlet fever patients, generally as the predominating organism and invariably early in the disease. (*b*) In severe scarlet fever infections and in fatal cases the organisms can often be found in blood cultures and in the heart's blood at autopsy. (*c*) Scarlet fever has been produced in non-immune volunteers with pure cultures of hemolytic streptococci. (*d*) Broth culture filtrates of these organisms produce, in high dilution, a characteristic local skin reaction and in larger doses a generalized rash and symptoms, when injected in susceptible individuals. (*e*) There is a close correlation between a positive skin reaction and susceptibility and between a negative reaction and immunity to the disease. The reaction is generally positive at the onset of the disease and becomes negative during convalescence. (*f*) Properly prepared immune sera and scarlet fever convalescent sera will neutralize the toxic filtrates for susceptible individuals. (*g*) Immune serum prepared with streptococci and their products will in early cases produce a local fading of the scarlet fever rash. (*h*) Therapeutic results in cases treated with serum made both by the Dochez and the Dick methods and by modifications of these have been favorable.

The Specificity of Scarlatinal Toxin. Dick and Dick (24) believed that hemolytic streptococci isolated from cases of scarlet fever produce a toxin different from that produced by streptococci isolated from other sources. They carried out a considerable number of neutralization tests in which pure strain sera were used and tested scarlet fever and erysipelas antitoxin against twenty-five scarlet fever toxins and three strong erysipelas toxins. They reported that the three erysipelas toxins were neutralized by the erysipelas antitoxin in all of twelve subjects on which they were tested and were not in any instance neutralized by the scarlet

fever antitoxin. They adhered to their idea of immunological specificity by further stating that an attack of scarlet fever does not confer immunity to erysipelas, that immunization with scarlet fever toxin does not confer immunity to erysipelas toxin, and that erysipelas antitoxin produced with a single erysipelas strain specifically neutralizes the toxins from other strains of erysipelas streptococci but does not neutralize scarlet fever toxins. We might point out at this time that scarlatinal antitoxic immunity does not protect against other septic streptococcus infections such as puerperal sepsis, cellulitis, meningitis, or mastoiditis, and all such infections are frequently of scarlatinal origin.

We now have abundant experimental evidence to refute this opinion of the Dicks. Kirkbride and Wheeler (25, 26) and Kirkbride, Wheeler, and Hendry (27) found that the toxins of streptococci obtained from different sources were often similar to each other and that it was difficult or impossible to differentiate organisms from different sources into special groups. Park and Spiegel (28) obtained results which indicated that an individual convalescent serum might neutralize the toxins of streptococci from a number of different sources to a greater or less degree. Wadsworth and his co-workers (29, 30) found that the toxic action of 85 per cent of 200 strains of hemolytic streptococci from scarlet fever and 70 per cent of 200 strains from other sources was quite similar when tested in goats. Approximately 65 per cent of all these toxins, irrespective of whether the source of the strains was a scarlet fever or non-scarlet fever infection, were neutralized by a control antitoxic serum produced by the single strain, Dochez No. 5. Similar to this in significance are the conclusions of Williams (31). Additional studies by Lash (32), Eagles (33), Smith (34), McLachlan (35), and Okell and Parish (36) provide abundant evidence for concluding that the production of an erythrogenic toxin capable of eliciting an erythematous skin reaction in man and which is neutralized by either scarlet fever convalescent serum or horse antitoxin is a property of hemolytic streptococci of human origin in general. While different strains vary in the amount of toxin produced and some do not produce toxin, we must conclude that there is no such thing as a specific scarlatinal toxin or a specific erysipelas toxin.

Hooker and Follensby (37) and Hooker (38) obtained evidence that there is more than one antigenic component in the erythrogenic toxin produced by hemolytic streptococci. Their experiments indicate the existence of two such toxins, A and B. Some strains of hemolytic streptococci such as the Dochez N.Y. 5 produce both toxins. Other strains may produce only one or neither. These findings have been confirmed by Cooke (39). The importance of these observations is that they explain some of the recorded clinical discrepancies concerning the use of Dick toxin and antitoxin. For example, Joe (40) has reported indi-

viduals with a negative Dick reaction at the beginning of scarlet fever. Others had a positive reaction in the convalescent period when their serum contained antitoxin. McGibbon (41) described similar anomalous features of the Dick reaction. About one fourth of a series of 160 cases were Dick negative during the acute stage of the disease. Trask and Blake's (42) observations suggest the existence of more than one toxin. The sera of two fatal cases of scarlet fever and the pleural exudate of a third contained erythrogenic toxin that was not neutralized by standard Dick antitoxin. All three patients had failed to respond to treatment with antitoxin. Toxin, prepared from the streptococcus isolated from one of the cases, was compared with standard Dick toxin in tests on 170 individuals. Ninety-three subjects reacted positively to Dick toxin and of these 33 failed to react to the new toxin. Of 78 subjects with a negative reaction to standard Dick toxin, 36 failed to react to the new toxin and 42 gave a positive reaction. Okell (43) noted 22 Dick-positive reactors among 119 cases of scarlet fever 3 to 12 weeks after the onset of the disease. He suggested that many individuals who must be immune because of having had scarlet fever in early life give positive reactions. On the basis of Hooker's observations we would suggest that such individuals may be immune to one type of toxin and not to others. Therefore they cannot properly be said to be immune to scarlet fever.

The Types of Hemolytic Streptococci Associated with Scarlet Fever. We have already mentioned the early studies of Moser and von Pirquet and others which suggested that streptococci of scarlatinal origin tended to fall into a particular serological group. This question was again reopened in 1919 by Dochez, Avery, and Lancefield (44), who found that most hemolytic streptococci isolated from a variety of sources formed at least six biological types. Additional studies by Bliss (45, 46), Tunnicliff (47), Gordon (48), Eagles (49), and Stevens and Dochez (50, 51) also suggested that hemolytic streptococci of scarlet fever origin tended to fall into limited serological groups.

The studies of Griffith (52, 53, 54) and of Lancefield (55, 56, 57, 58, 59) based upon antigenic analysis by more precise methods have shown that hemolytic streptococci of human origin can be divided into some 27 distinct serological types. All these types form a common group, the Group A of Lancefield or *Str. pyogenes* of Griffith. Studies of Griffith, Smith (34), Gunn and Griffith (60), McLachlan and Mackie (61), and others show that no single antigenic type is common to scarlet fever. While Griffith's types 1, 2, 3, and 4 are more commonly found in cases of scarlet fever, nevertheless, many other types of Group A streptococci have been associated with the disease.

Thus, it is clear that no one type of hemolytic streptococcus is the specific cause of scarlet fever. All such terms as *H. streptococcus scar-*

latinae or *Str. pyogenes* var. *scarlatinae* should be discarded. Any hemolytic streptococcus, regardless of its type, capable of producing erythrogenic toxin may cause scarlet fever in a susceptible nost. Certain host factors such as the presence or absence of antitoxic immunity determine whether or not scarlet fever will develop in an individual adequately infected with a toxin-producing strain of the organism.

The Schultz-Charlton Phenomenon. In 1918 Schultz and Charlton (62) described a reaction which is now commonly referred to as the Schultz-Charlton phenomenon, as well as by other descriptive terms such as the "blanching phenomenon" and the "rash extinction test." They injected 1.0 cc. of serum from a normal individual into the skin of a patient with a typical scarlet fever rash. This produced a fading of the rash in the immediate neighborhood of the injection. This property was shown to be caused by the serum of patients convalescent from scarlet fever but not by the serum of patients taken during the height of the disease. This phenomenon was at first incorrectly interpreted as being due to some substance present in normal serum that was removed during an attack of scarlet fever. Further studies of the mechanism of production of this reaction contributed important information toward our understanding of the etiology of scarlet fever.

Mair (63), in 1923, made significant observations concerning the blanching phenomenon. He had an opportunity to demonstrate that the serum of a child gave a negative reaction before an attack of scarlet fever and a positive reaction after convalescence. The sera of children who had not had the disease gave a much higher percentage of negative reactions than the sera of a random sample of adults. Although scarlatinal toxin had not yet been described, Mair came to the conclusion, definitely substantiated by later research, that the serum of those giving a positive rash extinction test contained antitoxin capable of neutralizing scarlet fever toxin. He made the prediction that the true causal organism when discovered should be capable of producing a toxin, and that immunization of animals with such a toxin should give rise to the development of antitoxin capable of producing a positive rash extinction test in man. Blake, Trask, and Lynch proved this prediction with Dochez's serum.

Mair believed that scarlatinal toxin combined with tissue cells, among which were the contractile elements in capillary vessels. The interference with the function of these cells, resulting in a loss of tone, was responsible for the production of the erythematous rash. He suggested that antitoxin dislodged this toxin, restoring normal function to the tissues. Von Bormann and Wolff-Eisner (64) contributed a detailed study of this reaction and concluded that it depends upon a true local neutralization of the toxin in the skin.

The Schultz-Charlton reaction is used extensively as a diagnostic test, particularly as an aid in the differential diagnosis of scarlet fever from clinically similar rashes such as in German measles and certain drug idiosyncrasies. The test consists of the intradermal injection of 0.5 cc. of convalescent scarlet fever serum or 0.1 cc. of a potent scarlatinal antitoxin produced in horses. The injection is made in an area of bright red rash, the skin of the abdomen usually being a suitable site. In a positive test blanching usually begins to appear after 6 or 8 hours. It may appear at any time up to 14 hours. The blanched area is usually one-half to a few inches in diameter and the margin is often fairly well defined, particularly when viewed from a short distance. The swelling of the follicles in the area may disappear, with the skin assuming a normal color and appearance. The blanched appearance usually lasts throughout the duration of the rash.

There are definite limitations to the use of the rash extinction test in the diagnosis of scarlet fever. The absence of a positive reaction does not negative the diagnosis. Mair and others pointed out that it is often unsatisfactory in coarsely punctate rashes and works better in those that are diffusely erythematous. Its value progressively diminishes with aging of the rash. Blake (65), in England, observed that it was positive in 83.3 per cent of cases on the first day of the rash, 75 per cent on the second day, and only 50 per cent on the third day.

Bacterial Allergy in Scarlet Fever. Bristol (66), Cooke (67, 68, 69, 70, 71, 72, 73), Dochez and Sherman (74), and Dochez and Stevens (75) have presented experimental evidence which suggests the possibility that the rash of scarlet fever as well as some of the skin reactions obtained with Dick toxin may be allergic in origin. Zinsser and Grinnell (76) and Dochez and Sherman demonstrated allergic reactions in animals sensitized to streptococci. Cooke made the observation that in early infancy Dick tests are usually negative and that the serum of such negatively reacting children has no neutralizing power. The supposition made was that repeated infection with hemolytic streptococci may sensitize certain individuals who, on a subsequent severe hemolytic streptococcus infection, respond with a clinical picture modified by their existing allergic condition.

Zingher (77), in 1924, showed that some individuals reacted to protein products of the streptococcus other than toxin. He pointed out that such pseudo reactions were analogous to those following the Schick test, and introduced as a control scarlatinal toxin that was heated to a boiling temperature for one hour. This problem was further clarified by Ando and his associates (78, 79, 80, 81), who found that there is a relatively heat-labile soluble exotoxin in streptococcus filtrates to which white pigs are susceptible and which can be precipitated out of the crude toxin with

two volumes of absolute alcohol. In addition to this they demonstrated a streptococcus nucleoprotein substance which is responsible for allergic reactions such as those observed by Zinsser and Grinnell and by Dochez and Sherman. This nucleoprotein of the hemolytic streptococcus is responsible for the production of the pseudo Dick reaction.

A false interpretation of the immunity state because of pseudo reactions can be avoided by using a control consisting of diluted toxin which has been autoclaved for one hour at 120° C. The pseudo reaction is smaller and less intense than a true positive reaction and fades more slowly. Thus the time relationships between a positive and pseudo reaction in the Dick test are the reverse of those in the Schick test.

We agree with Hooker (82) that all the clinical manifestations of scarlet fever except the septic complications can be explained by the soluble toxin and that allergy plays no role in their production. The rash, toxemia, and other systemic manifestations are due to the soluble toxin produced in the local lesion and absorbed into the circulation in much the same manner as in diphtheria. Experiments of Trask and Blake (83) are of much interest in this connection. They demonstrated a toxic substance in the circulating blood and urine during the acute stage of scarlet fever which caused an erythematous reaction when injected intracutaneously, in individuals whose serum gave a negative Schultz-Charlton reaction, *i.e.*, contained no antitoxin. This substance was present in the circulating blood for several days and rapidly disappeared following the injection of a large amount of antitoxin.

Production of Erythrogenic Toxin. For the production of potent toxin it is, of course, essential to use an active, toxigenic strain of hemolytic streptococcus. The so-called Dochez N.Y. 5 strain is the one commonly employed in this country. For production of material for susceptibility tests Dick and Dick employ three strains, labeled I, II, and III.

Various media have been used in the production of erythrogenic toxin. For preparing Dick-testing material the Scarlet Fever Commission requires the growth of their three strains in a buffered medium containing Witte's peptone and Liebig's meat extract. Malcolm and Wyman (84) used a modified veal infusion broth similar to that for diphtheria toxin production. Rane and Wyman (85) used a modification of Wadsworth and Wheeler's (86) infusion-free peptone medium. Veldee (87), in his recent study, employed a three-quarter strength Douglas tryptic digest broth.

An important contribution for the production of maximal growth of streptococci was made by Mueller and Klise (88), who added 2 per cent glucose to the medium and kept the reaction properly adjusted by the frequent addition of sterile sodium hydroxide. Hooker (89) and his associates employed this method for producing erythrogenic toxin.

Using the Dochez N.Y. 5 strain, they obtained toxin containing 500,000 Skin Test Doses per cc. in 24 hours' incubation. A value of 300,000 S.T.D. was obtained on the same medium without adjusting the reaction. Without the addition of glucose and without the adjustment of the reaction the value was 100,000 S.T.D. per cc. Methods employing these principles are now generally used for toxin production.

Standardization of Scarlet Fever Products. The standardization of scarlet fever toxin and antitoxin is beset with many difficulties. Titrations are most commonly done, at the insistence of the Dicks, on susceptible human beings. There is, of course, considerable variation in the susceptibility of positive Dick reacting individuals which necessitates the use of a large group for a particular titration. The practical difficulties in obtaining suitable persons for such tests are, of course, apparent. The same reasoning is applicable to standardization of antitoxin in human subjects. In this connection one cannot help but wonder how often an individual has been sensitized to horse serum by the latter procedure.

Many attempts have been made to standardize these products in various animals. Rosenow (90) and Ando and Kurauchi (91, 92) used white pigs for this purpose. Wadsworth, Kirkbride, and Wheeler (93. 94, 95) used goats. During the past few years various methods have been employed in chinchilla rabbits, and some of these give satisfactory results.

For purposes of standardization it is necessary to have definitely defined units of substances to work with.

The unit of scarlatinal toxin is the skin test dose, which is defined as that amount of toxin which will cause a reaction at least 1 centimeter in diameter following its intracutaneous injection in a majority of susceptible individuals. In titrating antitoxin five skin test doses are employed as a test dose. Dyer (96) has defined the unit of antitoxin as being 10 times the smallest amount of antitoxin necessary to neutralize one test dose of toxin. Thus, a unit of antitoxin is that amount which will neutralize 50 S.T.D. of toxin. Dyer further suggested that the designated amount of serum shall be the final measurement of reference, *i.e.*, the unit of serum will remain the same without regard to the dose of toxin used from time to time. Comparison, therefore, is to be made with the standard antitoxin provided by the National Institute of Health. This standard serum is provided already diluted, so that each cubic centimeter contains 40 such units. (The Permanent Commission on the Standardization of Sera, etc., of the Health Organization of the League of Nations selected as a basis for study the unit of antitoxin described by Dyer and the standard antitoxin provided by the National Institute of Health.)

Standardization of Toxin [Method of Dick and Dick (97)]. "To standardize the toxin 1 cc. is placed in a sterile 1-liter volumetric flask, and diluted to 1000 cc. with sterile physiologic sodium chloride solution. Another 1 cc. is made up to 2000 cc. with sterile salt solution. Skin tests are made with 0.1 cc. of each dilution on one arm of a susceptible person, and a test is made on the other arm of the same person with 0.1 cc. of the standard skin test solution (their own standard). The reactions are observed at the end of 24 hours. If the reaction with the 1 : 1000 dilution of the new toxin is weaker than the reaction with the standard skin test solution, the entire lot of toxin is discarded.

"If the reaction with the 1 : 2000 dilution is stronger than that of the standard skin test solution, similar tests are made with higher dilutions. If the 1 : 2000 dilution gives a weaker reaction, while the 1 : 1000 test is stronger than that of the standard solution a dilution of 1 : 1500 is tried.

"Various dilutions are made until one is found that gives reactions corresponding exactly to those obtained with the standard solution. This dilution is then checked against the standard solution in a series of at least 20 susceptible and 10 immune individuals. The positive tests should include slightly, moderately, and strongly positive reactions. Records of the reactions are made showing their measurements in millimeters in two diameters, the intensity of color, and the presence or absence of swelling. Tests in slightly susceptible persons sometimes reveal errors in standardization that are not apparent in the more strongly positive reactions."

Standardization of Antitoxin [Method of Dick and Dick (98)]. "For standardizing, the concentrated antitoxin is diluted 1 to 10 in sterile, physiologic sodium chloride solution. Two cubic centimeters of this diluted antitoxin are mixed with 2 cc. of a toxin solution. The solution of toxin used is 10 times as strong as the standard skin test solution, so that each cubic centimeter represents 100 skin test doses.

"Two control solutions are made, one of equal parts of toxin and salt solution, the other of equal parts of the diluted antitoxin and salt solution.

"These three mixtures are incubated one hour. Skin tests are then made with 0.1 cc. of each mixture in persons with positive to strongly positive skin reactions. Persons with slightly positive reactions are not acceptable for these tests.

"On observation, at the end of 24 hours, the test made with the antitoxin alone should be negative. If this is positive, the person is sensitive to horse serum, and is not useful for the standardization. The test with the toxin alone should be positive. The test with the toxin-antitoxin mixture will be negative if the antitoxin is sufficiently strong.

"At the end of 48 hours, another observation is made. The weaker antitoxins, like convalescent serums, may hold the toxin in combination 24 hours, but release it after that; so that the toxin-antitoxin test that was negative at the end of 24 hours may be found positive at the end of 48 hours.

"For further standardization, the same tests are repeated with higher dilutions of the antitoxin."

Standardization of Antitoxin by the Rabbit Ear Method of Veldee (99). "A sufficient number of reacting animals is obtained by injecting a considerable number of fresh rabbits with one human skin test dose of purified and concentrated toxin contained in 0.1 cc. volume, a day or more in advance of putting on the neutralization test. Several days in advance is preferable,

so that the reaction produced, especially on the more susceptible animals, will have subsided somewhat before using for the neutralization tests. The preliminary toxin-injection is made intradermally on the inside of the right pinna in the mid-line and near the base. This leaves the remainder of the right ear flap and all of the left for the toxin-antitoxin tests. Readings are made in 18 to 24 hours, and only those rabbits are reserved for test purposes which show a reaction area of 10 by 10 mm. or more in dimension and which show an intensity of + when measured on a scale of 0, ±, +, and ++.

"The toxin-antitoxin mixtures are made in the usual way, using 25 human skin test doses of purified and concentrated toxin as the unit of toxin in each instance instead of 5 S.T.D. as are used for the human test dose. The mixtures are placed at 37° C. for one hour. The injections of 0.1 cc. each are made on the inner side of the pinna between the main blood vessels and the margin of the ear. The size of the ear flap permits three injections equally spaced on the right and the same number in corresponding positions on the left, with an additional injection on the left in a position corresponding to the site of the test toxin injection on the right. The usual Schick syringe and needles may be used, though the writer prefers a 26-gauge, one-half inch, flexible shaft and a long-beveled needle in preference to the usually used short, blunt Schick needle. The ear flap is supported on the fingers of the left hand so as to expose the ventral surface. When the needle has been inserted into the skin directly over the left index finger, the left thumb is pressed down on the ear at the junction of the needle shaft and the skin of the ear. This will hold the needle securely in place between thumb and index finger in case the rabbit struggles, and it will also prevent leakage along the needle shaft. The needle is withdrawn while the thumb is still in place. The skin on the inside of the ear possesses very little elasticity, and for this reason leakage is likely to occur when the needle is withdrawn. This leakage may be avoided by rubbing a small amount of collodion into the needle hole.

"Readings are made at 24 and 48 hours, though it is advisable to make an observation at 15 to 18 hours as well. This gives a better impression of the development of the reaction. It will be observed that when the ear is viewed by reflected light nothing abnormal is seen unless the reaction is of pronounced intensity. The readings must, therefore, be made by light transmitted through the pinna. This should be good daylight or its equivalent, direct sunlight or the usual electric light being unsatisfactory. Readings are made with the rabbit sitting quietly and without unnecessary manipulation of the ear. Gentle massage will increase the intensity of the reaction, but will not render a negative reaction positive. However, it is believed that reading the undisturbed ear is preferable.

"Two dimensions of the inflamed area are recorded and also the intensity. The intensity is recorded as ±, +, or ++. In the present work, reactions of less than 10 by 10 mm. in area were considered as negative. With further experience, however, it may be advisable to regard any reaction other than the trauma caused by the needle insertion as a positive reaction."

Rabbit Intradermal Method. Fraser and Plummer (100) in 1930 found that satisfactory titrations could be done in the skin of the back and flanks of chinchilla rabbits. These observations were confirmed by

Kolchin (101). Buttle and Lowdon (102) found that this method of testing was reliable within a 1:2 ratio.

Rabbit intradermal methods have been employed for a number of years at the Massachusetts State Antitoxin Laboratory. Their methods as described by Malcolm and Wyman (103) are as follows:

"The reacting rabbits were selected by injecting fresh animals subcutaneously with 1 human S.T.D. of toxin in a 0.1 cc. volume on the inside of the ear near the base of the lobe. Readings were made in 24 hours, and those rabbits which showed a reaction of 10 by 10 mm. or greater with a pink intensity were used for test purposes. Animals selected by this means are closely clipped over the back, mid-way down the sides and marked in squares of approximately one inch. A total of 50 to 60 injections can be made on the skin and ears of a large rabbit without crowding. Large mature rabbits are the most suitable if not essential for intradermal tests, as their skin is firmer than that of young animals and the reactions more definite.

Titration of Toxins. "We have found that the ear is much superior to the body skin for titration of toxins. Determination in the skin of the amount of toxin neutralized by a given amount of antitoxin gives an indication of the probable S.T.D. content of the toxin. For this reason, the approximate S.T.D. content is first estimated by means of skin neutralization tests and this result is verified by testing closer dilutions of the toxin on the rabbit's ears. This permits the use of a smaller number of rabbits than if all titrations were done with toxin alone.

"A standard toxin of known value is diluted so that 1 cc. contains 500 S.T.D. Similarly, an appropriate series of dilutions of the untitrated toxin are so made that there is not more than a 25 per cent interval between each dilution. A standard scarlet fever streptococcus antitoxin is used and toxin-antitoxin mixtures made with both the standard and untitrated toxins in the same manner as in the neutralization test, using the standard toxin plus antitoxin mixture as a control. An approximate titer of the toxin may be obtained by merely determining which dilution of the unstandardized toxin-antitoxin mixture gives approximately the same end-point as the standardized mixture, and correcting for the difference in the dilutions of the two toxins.

"After the establishment of approximate values by means of the neutralization test, it is necessary to use the ear for accurate titration. This is obtained by comparing the size and intensity of the reaction of the untitrated toxin dilutions in one ear, with that of 1 S.T.D. of standard toxin in the other ear. When there are no discernible differences in the reactivity of the two toxins by means of the ear test, a comparative test is made on a few human subjects for the final standardization procedure."

Titrations of Antitoxins. "The method as outlined by Veldee, in the ear test, with regard to the selection of animals and the test dose of toxin has been closely followed in the titration of antitoxins. The toxin used may be either crude or concentrated. It is diluted so that 1.0 cc. contains 500 human S.T.D. A series of dilutions of standard scarlet fever streptococcus antitoxin furnished by the National Institute of Health are so made that 1.0 cc. contains 20, 40, 60, 80, and 100 neutralizing skin test doses, respectively. The antitoxins to be tested are appropriately diluted, each dilution containing usually one-half and a minimum of one-quarter as much anti-

toxin as the preceding. There is now added to 2 cc. of each of the antitoxin dilutions 2 cc. of the diluted toxin and the resulting mixtures incubated for one hour at 37° C.

"The volume injected is 0.1 cc., which contains 25 S.T.D. of toxin plus antitoxin. With the ear test one ear is used for the test mixtures and the other ear for the control mixtures. Standard control antitoxin-toxin mixtures are similarly used with the skin test method. With the latter method the size of the intradermal reaction is not recorded in terms of millimeters but instead as positive (+) or negative (−). The size of a positive skin reaction, although recorded only as +, is usually at least 10 by 10 mm. with a pink or faint pink intensity, but this is variable. The end-point is considered as that dilution where no reaction occurs. Reactions on the skin appear more prominently in artificial light, whereas those in the ear are distinguished by good daylight."

Standardization by Flocculation. Many investigators, including Dyer (104), Povitzky (105, 106), Eagles (107), Ramon, Fraser, Martin, and Laffaille (108), O'Brien, Okell, and Parish (109), Truschina, Krestownikowa, and Loginowskaja (110), and O'Meara (111) have attempted to standardize scarlet fever toxin and antitoxin by means of the flocculation reaction. These investigations met with indifferent success in establishing a correlation between the *in vivo* and *in vitro* titrations.

Rane and Wyman (85) have recently succeeded in getting true flocculation reactions by using very strong toxins. They found that one unit of antitoxin flocculates with approximately 60,000 S.T.D. instead of the 50 S.T.D. to be expected. This fact probably explains the lack of success by the earlier investigators.

Rane and Wyman produced their toxin by growing the organism in a simple, peptone-free medium. By frequent additions of glucose and keeping the reaction adjusted by additions of sodium hydroxide they obtained toxins of 120,000 to 180,000 S.T.D. per cc. This crude toxin was concentrated by ammonium sulfate precipitation in the following manner: 438 grams of solid ammonium sulfate were dissolved in each liter of chilled toxin and held overnight at 4° C. The precipitate was collected by centrifugation and dialyzed against running tap water. The dialyzed solution was chilled and brought to *p*H 4.0 to 4.2 with glacial acetic acid. After standing in the cold for one-half hour the precipitate was removed by centrifuging and discarded. The supernatant liquid, containing the toxin, was adjusted to *p*H 7.0 with sodium hydroxide. This concentration process removed about 90 per cent of the original nitrogen. These concentrated toxins contained from 2,400,000 to 3,600,000 S.T.D. per cc. and yielded 40 to 60 flocculating units per cc.

We quote the description of Rane and Wyman's flocculation reaction:

"**Technique of the Flocculation Test.** The antitoxin is placed in a series of tubes in amounts of 0.10 to 0.20 cc., varying by 0.01 cc. per tube, and the

toxin (1.0 cc.) added; and after the two are thoroughly mixed by inverting the tubes three or four times, the tubes are placed in a water-bath with only one-third of the columns of liquid immersed, in order to promote convection currents. The slight differences in the total volumes in the tubes are not sufficient to affect the accuracy of the readings. Table I illustrates a typical titration of an unknown concentrated toxin.

TABLE I

TYPICAL FLOCCULATION TEST

Concentrated toxin. Lot XXIX. Standard serum 629. 240 units (by rabbit-test). Temperature of water-bath, 42° C.

	Tube 1	Tube 2	Tube 3	Tube 4	Tube 5	Tube 6	Tube 7	Tube 8	Tube 9	Tube 10
Standard serum . . .	0.11	0.12	0.13	0.14	0.15	0.16	0.17	0.18	0.19	0.20
Toxin, cc.	1	1	1	1	1	1	1	1	1	1
Reading in 5 minutes .			C	C	C	C	C	C	C	C
Reading in 15 minutes .						F				

C = cloudy. F = flocculation.

The L_f dose of the toxin =

$$\frac{1.0 \text{ (volume toxin)}}{0.16 \text{ (quantity of serum)} \times 240 \text{ (antitoxic titer)}} = 0.026 \text{ cc.}$$

or 1 cc. of toxin contains 38.4 L_f units, as defined by Glenny and Okell (112), the amount of toxin established by flocculation to be equivalent to one unit of standard antitoxin.

Taken from Rane and Wyman, *loc. cit.*

"In 15 minutes small floccules were visible in one tube, and they gradually increased in size to heavy flakes and settled to the bottom of the tube. From the tube giving the first evidence of flocculation the potency of the toxin can be calculated.

"**Titrations of Toxins.** For titrating toxins, a serum has been carefully standardized by the intracutaneous method in rabbits. It contains per cubic centimeter 240 units (National Institute of Health), which are equivalent to 12,000 of the Scarlet Fever Committee original neutralizing skin-test doses.

"The strength of a toxin, if sufficiently great, may be evaluated by flocculation of the standard serum directly, or, in the case of weak toxins or of those of unknown potency, by blending (generally in equal volumes) with a toxin of known L_f value. When the L_f value of the blend has been established, the range at which the unknown toxin can be titrated directly is easily determined. As with the Ramon reaction, blending gives accurate values and is useful in dealing with weak or slowly flocculating toxins or with toxins whose value cannot be estimated before testing.

"The L_f value of a weak toxin can usually be determined without blending but prolonged incubation is required and the amount of precipitate formed may be so small that great care must be exercised in reading the reactions.

"**Titration of Antitoxins.** For titrating antitoxins, a concentrated toxin of known L_f value is employed in amounts of 1.0 cc. per tube. The antitoxin is used either undiluted, or diluted so that one of the doses employed will contain about 40 units in about 0.10 to 0.20 cc. When the value of the antitoxin is entirely unknown, a reasonably accurate measure of its potency can be obtained by blending the unknown serum with the standard serum and titrating the mixture. The unknown antitoxin or a suitably different blend of the two antitoxins may then be titrated more closely. By these methods satisfactory tests can be made with serums containing as little as 10 units of antitoxin per cubic centimeter."

The Dick Test. This test is performed by injecting intracutaneously 0.1 cc. of standard test toxin, this amount containing 1 skin test dose of toxin. A positive reaction consists of an area of erythema at least 1 cm. in diameter appearing in 18 to 24 hours and fading rather rapidly. A control test should always be done consisting of a similar injection of the same test material which has been heated sufficiently to destroy the relatively heat-resistant erythrogenic toxin. Pseudo reactions due to sensitivity to bacterial protein appear in certain individuals. Such reactions are more common with advancing age. The pseudo reaction is also an area of erythema which may or may not exhibit some induration. This reaction appears later and fades more slowly than the true positive reaction.

There is a striking analogy between scarlet fever and diphtheria in the distribution of natural immunity in any population. Zingher (77) reported a large number of observations on individuals of various ages. His figures, presented in Table II, are in essential agreement with those subsequently reported.

TABLE II

THE DICK TEST IN DIFFERENT AGE GROUPS

AGE	TOTAL TESTED	DICK POSITIVE	DICK NEGATIVE	PER CENT DICK POSITIVE
0–6 months	29	13	16	44.8
6–12 months	42	27	15	64.2
1–2 years	123	87	36	70.7
2–3 years	140	95	45	67.7
3–4 years	207	123	84	59.4
4–5 years	237	110	127	46.4
5–10 years	1475	522	953	35.4
10–15 years	1690	430	1260	25.4
15–20 years	285	75	210	26.3
Over 20	342	61	281	17.9
	4570	1543	3027	34.4

Taken from Zingher (77).

Thus, as indicated by this test, young infants are immune to scarlet fever. This immunity wanes during the second half of the first year, leaving the child susceptible to the disease. The greatest period of susceptibility is from the first to the fourth year of age. Thereafter the percentage of immunes gradually increases. In this connection one might again refer to the observation of Cooke, who showed that while young infants fail to react to the Dick test, he was unable to demonstrate antitoxin in their blood. Toomey and August (113) investigated the presence of antitoxin in placental serum and found that 79 per cent of 227 specimens examined had the property of blanching the rash of scarlet fever, a function which we attribute to the presence of antitoxin. Furthermore, McKhann and Chu (114) showed that substances capable of blanching the rash of scarlet fever were present in their preparations of placental extract. In the latter experiments, however, the extracts were prepared from the whole organ and therefore contained maternal as well as fetal products.

When one considers the distribution of natural immunity to scarlet fever in relation to the various factors which might influence it, the resemblance to the distribution of natural immunity to diphtheria is again quite evident. Zingher found a great difference in the susceptibility to the disease among different children according to their economic status. Children of the same age in the economically well-to-do classes showed a much greater susceptibility rate than children from less fortunate homes. This investigator, as well as Dyer, Caton, and Sockrider (115), showed that the susceptibility rate was much greater in urban populations than in rural. Furthermore, the distribution of susceptibility among different races and in different climates is similar to that in diphtheria. Van Slype (116) studied the response to the Dick test among natives in the Belgian Congo and found a high rate of immunity. Among 201 individuals tested more than 90 per cent were Dick negative. It was found that the percentage of negative reactions increased very rapidly after the first year of life. It was pointed out that scarlet fever is a rare disease in this country, although other types of streptococcal infection are not uncommon. Other studies have indicated a similar high rate of immunity in tropical countries, and it has long been known that the disease is relatively infrequent in such regions.

Sherwood, Nigg, and Baumgartner (117) studied the susceptibility of a group of American Indians, a race curiously free from scarlet fever. They found that in spite of the fact that the disease is seldom seen in this race, susceptibility among them, as determined by the skin test, was essentially equal to that among the whites in the younger ages, but was much less as maturity is reached. They felt there was some lack of parallelism between the skin test and natural immunity to scarlet fever.

The question of immediate concern to all is whether or not the Dick test is a reliable indication of the presence or absence of immunity to scarlet fever. The observations of the Dicks (118) would indicate that it is. They described rather impressive figures in support of this. They record 20,856 Dick-negative individuals who passed through one or more epidemics without contracting the disease. A group of 2157 Dick-negative nurses and internes who were frequently and intimately exposed to the disease failed to develop it.. On the other hand, numerous reports have appeared describing failures of this test. For example, Benson and Simpson (119) reported the development of scarlet fever in two nurses previously noted to be Dick negative. Buschmann (120) reported the development of scarlet fever in six Dick-negative individuals. McGibbon (41) reported an unusual finding, namely, that approximately one fourth of 160 patients appeared to be Dick negative in the acute stage of scarlet fever. This observation cannot, of course, completely invalidate the test because it is quite possible that in at least some of these patients antitoxin was already beginning to appear in an amount sufficient to cause a negative response to the test. Anderson and Reinhardt (121) studied the frequency of scarlet fever among the nurses in 38 hospitals in Massachusetts. They presented data on 6346 nurses and found that the attack rate was 12.8 per cent among those with positive reactions, whereas there was only 0.7 per cent among those with negative reactions. One must conclude that the Dick test gives fairly reliable information concerning immunity to scarlet fever. It would appear however that it does not have the same precision as the Schick test in diphtheria. There are various factors which might be responsible for the occasional failures of this test. One of the most important is the use of testing material of inferior potency. One of the factors responsible for the release of inferior products is the relatively unsatisfactory methods of standardization. The use of human beings for standardization, even though they are regarded as susceptible, is not precisely satisfactory because of the great variation in such susceptibility. Furthermore, it has been amply demonstrated that standardization only within relatively wide limits can be accomplished in laboratory animals. The wide variation in the potency of various lots of testing material was well shown in the studies of Friedman, Esserman, and Ginsberg (122). In addition, some of these anomalous responses may be due to the existence of more than one antigenic type of toxin. We have already mentioned the observations of Hooker and of Trask and Blake in this regard.

The Dick test has often been used as a diagnostic test in scarlet fever. It has a relatively limited usefulness for this purpose. If an individual exhibits a positive reaction in the beginning of an illness suspected of being scarlet fever and subsequently shows a negative response, it is

supportive evidence that the disease was scarlet fever. On the other hand, a negative reaction in the beginning of an illness does not rule out the possibility of scarlet fever, nor does the presence of a positive reaction during the convalescent period negative the diagnosis.

Active Immunization with Dick Toxin. With the demonstration of the production of a soluble toxin by hemolytic streptococci isolated from scarlet fever patients it was only natural that efforts were directed toward developing methods of active immunization. The Dicks were among the first to explore this field. The most successful method of immunization has been by subcutaneous injection of gradually increasing doses of toxin.

In the earlier studies there was a great deal of variation in the amount of toxin injected and the number of injections employed. The Dicks (97) at first thought that a relatively small amount would be adequate. They reported a reversal of the Dick reaction in 91.8 per cent of susceptible individuals following a total dosage of 10,000 to 12,500 S.T.D. In a later pronouncement they (118) recommended a much larger total dosage, consisting of five injections at weekly intervals commencing with 500 and increasing to 80,000 or 100,000 skin test doses. The resulting immunity was said to last three years in 90 per cent of those in whom the Dick reaction was rendered negative. A commonly used dosage schedule for this amount is as follows:

1st week = 500 S.T.D.
2d week = 2500 S.T.D.
3d week = 20,000 S.T.D.
4th week = 40,000 S.T.D.
5th week = 80,000 S.T.D.

Other investigators have used varying amounts of toxin; however, the tendency has been toward a larger total dosage. Smythe and Nesbit (123) found that a total of 50,000 S.T.D. rendered 66 per cent of susceptibles Dick negative, whereas an increase to 80,000 increased the negative rate to 85 per cent. Park and Schroeder (124) stated that at least 100,000 S.T.D. are necessary for the production of a prolonged immunity. Others have pointed out that the duration of the immunity is directly related to the total amount of toxin employed.

Immunization against scarlet fever has been most widely employed among the nurses, internes, and other personnel in contagious disease hospitals. For this purpose it would appear that the procedure is of considerable value. Numerous reports have appeared describing the results of immunization in institutions. In the City Fever Hospital at Liverpool, Stallybrass (125) noted that 11 nurses contracted the disease during the year prior to immunizing. After the introduction of active immunization only three cases occurred during a period of five years.

He also reports very favorable results from the immunization of the inmates of several institutions. Hannah (126) found a case rate of 3.9 for scarlet fever among the nurses of the Children's Hospital, Toronto, during the years 1918–1924. After the introduction of immunization the case rate has been 0.78 per 100 during the period 1925–1933. We have already noted the experience of Anderson and Reinhardt (121) in their study of scarlet fever among the nurses in 38 Massachusetts hospitals.

In spite of the reduction in the incidence of scarlet fever among nurses and institutional inmates, this does not occur without some cost. Many authors have pointed out that such immunization does not eliminate the individual as a source of infection to others. There is no doubt but that immune individuals may suffer from a streptococcus sore throat and not present clinical evidence of scarlet fever, *i.e.*, the so-called "scarlatina sine exanthem." Kinloch, Smith, and Taylor (23) reported an extensive experience in scarlet fever immunization. They found that there was an actual increase in the incidence of streptococcus tonsillitis among the immunized nurses. In most instances the responsible organism was found, by serological methods, to fall into one or the other of the scarlatinal types. On the other hand, Benson (127) found an actual decrease in the incidence of streptococcus tonsillitis. In this connection it is interesting to recall the early experiment of the Dicks in which the throats of five volunteers were swabbed with hemolytic streptococci. One of these developed typical scarlet fever, one developed acute tonsillitis, and no septic reaction occurred in the remaining three. Furthermore, the reactions to immunizing injections often cause loss of time from duty on the part of nurses and attendants. Benson noted the occurrence of constitutional symptoms in one third of a group of 78 nurses following first and second injections of 1000 and 5000 skin test doses. Rhoads (128) found a total loss from duty of 45.5 days among a group of 190 nurses, each of whom received a total of 115,500 skin test doses.

Numerous studies have been reported of large-scale immunization programs other than that in institutions. A few of these reports can be cited. Toyoda (129) and his associates, who have had an extensive experience with this problem in Dairen, reported that since immunization was begun the attack rate among the immunized was 1 per 1000, while among the non-immunized the rate was 43 per 1000. Sparrow (130) reported an incidence of 0.43 per cent among 21,955 immunized individuals, whereas the attack rate was 1.26 per cent among 89,188 non-immunized. Platou (131), Henry (132), Fraser and Fraser (133), and others have reported similar favorable results.

It has been the experience of all who have used raw Dick toxin for immunization against scarlet fever that reactions of varying degrees of

severity follow the injection of one or more of the doses of toxin. Local reactions occur in a large percentage of individuals. In addition a considerable number of individuals exhibit a generalized systemic reaction consisting of fever, headache, vomiting, and a scarlatiniform rash of varying intensity. Indeed, these reactions are often comparable in severity to the mild type of scarlet fever now generally prevailing in the United States, Canada, and Western Europe. This circumstance has been a factor in the failure to employ active immunization on a larger scale.

It is, of course, possible to reduce the number and severity of reactions by a longer and more graduated system of dosage. Rappoport (134) approached the final dose of 80,000 S.T.D. through a series of 10 injections starting with an initial dose of 150 S.T.D. However, the necessity for using so many injections is, in itself, a serious drawback. It is usually difficult enough to secure submission to a series of five injections, particularly if the first or second has been followed by a reaction. The necessity for multiple dosage is a handicap in a large-scale public health immunization program. The economic and administrative difficulties involved in addition to the failure to secure complete co-operation have been factors in restricting the widespread use of immunization.

As we have already pointed out, some consideration must be given to the septic as well as toxic aspects of the disease in any appraisal of the value of active immunization. There is no irrefutable immunologic reason or convincing epidemiologic data to indicate that active immunization with Dick toxin prevents septic infection with hemolytic streptococci capable of producing scarlet fever in a susceptible individual. Actually, the seriousness of the mild type of scarlatina that is being seen at the present time is very largely due to the septic complications which may occur.

In summary, we cannot recommend, for the various reasons discussed above, large-scale immunization as a part of a public health or community program. For the same reasons the private practitioner is not denying his private patients a valuable service by failing to immunize them with Dick toxin. On the other hand, there would appear to be some value in the use of this preventive procedure in the personnel of contagious disease hospitals and other institutions where the frequency and intimacy of exposure makes scarlet fever a more definite liability. At least the disruption of service due to quarantine and the economic loss for time off duty appears to be reduced by such immunization.

Duration of Active Immunity. The duration of immunity following immunization has been variously placed at from three to eight years. In this respect the immunity result is similar to that against diphtheria. Again, just as in diphtheria, the duration of active immunity cannot be

entirely credited to the immunization procedure. Subsequent contacts, and these must be fairly frequent, with hemolytic streptococci capable of producing erythrogenic toxin will result in secondary stimulation, a factor of great importance in the continuation of active immunity.

Active Immunization with Scarlatinal Toxoid. Soon after the discovery of erythrogenic toxin Zoeller (135, 136) attempted the production of toxoid by the use of Ramon's method. Since that time toxoid has been used by a number of investigators in an effort to produce active immunity against scarlet fever.

Ando and Ozaki (137) concentrated their toxoid by alcohol precipitation, which eliminated the formalin and most of the nucleoprotein. They pointed out that when the toxin was de-toxified to about $\frac{1}{1000}$ of its original toxicity, its immunizing value was considerably impaired. On the other hand, 80 per cent of children were successfully immunized with a preparation de-toxified to $\frac{1}{200}$ to $\frac{1}{300}$ of its original toxicity. Three intramuscular injections of a preparation corresponding to 10,000 S.T.D., 20,000 S.T.D., and 30,000 S.T.D. were employed. Olarin (138) carried out extensive immunizations with toxoid among children in a number of schools in Bucharest. Using two different preparations of toxoid he found that the Dick reaction was reversed in 77.15 per cent with one and 84.53 per cent with the other. Evidence was obtained to indicate that both the morbidity and mortality from the disease were less in the immunized children. It should be pointed out, however, that his material was not de-toxified to an appreciable extent; that is, its potency had been reduced to only about $\frac{1}{15}$ of its original value. Debré and Ramon (139) immunized 145 children with toxoid and reported that the Dick reaction became negative in only 65 per cent of the children.

Veldee (140, 141) has reported his experiences with the preparation and use of scarlatinal toxoid in this country. He concentrated his toxin by precipitation with acetone before treating it with formaldehyde. His concentrated toxin contained 200,000 S.T.D. per cc. This was treated with formalin until it contained no more than 500 S.T.D. per cc., this procedure requiring about 60 days' incubation. A large group of children were immunized by three injections of this material. Of 1168 persons Dick tested one month after the last injection, 83.2 per cent were Dick negative, and of 494 persons re-tested about 8 months after the last dose, 87 per cent were Dick negative. Veldee concluded from this as well as from tests in rabbits that his toxoid was definitely antigenic and that it was unlikely that the immunity was due to the small amount of residual toxin. The Dicks (142) obtained some of Veldee's toxoid and immunized a group of Dick-positive individuals with it. They found that only 40 per cent had become Dick negative a month later. They investigated the sample of toxoid and claimed that it contained 8000

S.T.D. of residual toxin per cc. and not 500 as was stated. The Dicks concluded, therefore, that there is no such thing as a scarlatinal toxoid, and ascribed the immunity of individuals in whom it was used to the residual toxin it contained.

Cantacuzène (143), with a very large experience with scarlatinal immunization in Rumania, concluded that toxoid was of very little value. He also criticized the value of the Dick test as well as other methods of active immunization and presented considerable epidemiological data in support of his opinions. Albesco (144, 145) also reported on the use of toxoid in a large number of immunizations in Bucharest. He compared the results obtained with toxoid with those secured by the use of raw toxin, and concluded that the toxoid was of definite value. It should be noted, however, that his toxoid was de-toxified only to $\frac{1}{15}$ to $\frac{1}{20}$ of its original potency. When one calculates the total dose of toxin in his toxoid and bears in mind the difficulties of accurate titrations, it is quite evident that there is no significant difference between his results obtained with toxoid and those with raw toxin. Hortopan and Ciulin (146) immunized a large group of children with a complete course of toxoid and found that of 529 of these subsequently Dick-tested only 57.5 per cent exhibited negative reactions. Furthermore, the preparations of toxoid used by them retained from 20 to 25 per cent of its original toxicity.

It is evident that the erythrogenic toxin of hemolytic streptococci does not react to the formol toxoiding process in a manner entirely analogous to diphtheria and tetanus toxins. In the first place, it is impossible completely to de-toxify such preparations. As a rule there is a residual toxin content of 250 to 500 S.T.D. per cc. in spite of a very prolonged period of incubation in the presence of formalin. We must agree with the Dicks that it is likely that the reported success from immunization with toxoid may be due to the residual toxin. As we have pointed out above, in many such studies, the toxin was not de-toxified to a minimum residual content. On the other hand, this does not mean, as the Dicks have claimed, that there is no such entity as a scarlatinal toxoid. While its antigenic power is undoubtedly considerably reduced, nevertheless it exhibits other immune reactions generally conceded to be characteristic of toxoids. For example, Rane and Wyman (85) have shown that scarlatinal toxoid flocculates with antitoxin to the same titer as the original raw toxin from which it was prepared.

Thus, there is very little evidence so far to indicate that scarlatinal toxoid is an effective immunizing agent. Furthermore, while reactions due to toxin *per se* are largely eliminated by the use of this material, nevertheless those due to bacterial nucleoprotein are still present. Indeed, the content of this material per immunizing dose is greater than in immunizing doses of raw toxin, since toxoid is an undiluted material.

We would like to indicate that there is an urgent need for a well-controlled study to convincingly demonstrate whether or not the immunity response to toxoid is due to the residual toxin content only. Such a study could be accomplished by the immunization of two groups of individuals with materials that have been assayed for toxin content with extreme care. One such group, of course, should receive the toxoid; the other group should receive corresponding amounts of toxin contained in an amount of the same broth used for toxin production equivalent to the dosage of toxoid. We feel that a study of this character when properly controlled would give us data of considerable value. Unfortunately, the Dick reaction is a qualitative test only for the follow-up appraisal of the immunity in such a study. One would like a quantitative measurement of the amount of immunity. The titration of antitoxin in the blood of immunized individuals is not sufficiently accurate or reliable for a study of this kind. It is possible that some quantitative information could be obtained by testing with multiple skin test doses of toxin in a manner similar to that used by Nemhauser (147).

Active Immunization by Other Methods. Veldee (148) has recently described another method of active immunization that would appear to minimize certain disadvantages of the older methods. His toxin was prepared by growing the N. Y. 5 strain in a broth prepared from human placental tissue instead of veal or beef. It was felt that this would eliminate certain undesirable foreign proteins. Maximum toxin production was obtained by keeping the reaction adjusted by frequent additions of sterile sodium hydroxide throughout the period of active growth. After filtration the toxin was precipitated by the addition of 3.5 volumes of 95 per cent ethyl alcohol. The precipitated toxin was redissolved in physiologic saline. Glacial acetic acid was added to this to a concentration of 1 per cent and the material placed in a cold room overnight. The resulting precipitate, consisting almost entirely of bacterial nucleoprotein, was discarded and the supernatant liquid, containing the erythrogenic toxin, was adjusted to the proper reaction. It was found that practically 100 per cent of the purified toxin could be removed in the form of an insoluble precipitate by the addition of tannic acid to a concentration of 0.5 per cent. A stabilized suspension of the precipitate was prepared by the addition of gum Acacia to 1 per cent.

The precipitated toxin was carefully standardized so that each of three 0.1 cc. doses contained 750, 3000, and 10,000 skin test doses. A course of immunization consisted of the intradermal injection of these three dosages at an interval of two weeks between injections. This system of dosage resulted in the reversal of the Dick reaction in over 80 per cent of Dick-positive children.

This procedure resulted in relatively few reactions, partly because of the intracutaneous route of injection and partly because of the use of purified material containing relatively little bacterial nucleoprotein. The insolubility of the finished product retards its absorption and lengthens the period of antigenic stimulation. It is, of course, a distinct advantage to be able to accomplish satisfactory immunization by the use of three injections instead of five or more.

Other methods of immunization have been proposed from time to time but none of these have been accorded general favor. For example, Colby (149) attempted to de-toxify scarlatinal toxin with sodium ricinoleate. Peters and Allison (150) made topical applications of toxin with a nasal spray and Kuroi (151) swabbed the toxin directly on the tonsils. The Dicks (152) claimed that antitoxin production will occur following the administration of toxin by mouth. Cooke's (153) observations indicated that there might be some antigenic stimulation following rectal instillation of toxin.

The Use of Antitoxin in the Treatment of Scarlet Fever. With the establishment by Dyer (96) of a definite standard unit of scarlatinal antitoxin, some order was given to the dosages to employ in the treatment of this disease. In the earlier literature on the subject dosage was usually described in terms of cubic centimeters of serum. This, of course, was extremely inaccurate because of the great variation in the potency of different preparations. Dyer felt it would be convenient to describe dosage in terms of units similar in amount to those employed in the treatment of diphtheria. It was generally agreed that an amount of antitoxin capable of neutralizing from 300,000 to 600,000 S. T. D. was a proper therapeutic dose. Dyer, therefore, defined the unit as the least amount of antitoxin required to neutralize 10 test doses of toxin, each test dose consisting of 5 skin test doses. Thus, the therapeutic doses generally employed at the present time vary from 6000 to 12,000 units of antitoxin, the actual amount depending on the severity of the disease in each individual case. The National Institute of Health requires that no antitoxin be dispensed unless it contains at least 400 units per cubic centimeter. Many products have been prepared, of course, with a considerably higher value than this. The antitoxin is generally administered by intramuscular injection, although it may be given intravenously.

It is difficult to discuss the therapeutic value of scarlatinal antitoxin largely because of the great differences of opinion concerning it. Indeed the fact that there is such a difference of opinion in itself casts a legitimate doubt upon the value of the procedure. It is best to discuss this question with reference to different specific types and phases of the disease.

In severe toxic cases it has been possible, as Trask and Blake showed,

to demonstrate erythrogenic toxin in the blood, urine, and other body fluids. It would appear that such cases should provide a critical test of the value of antitoxin. There have been a number of reports concerning the use of serum in such cases. Mersŏl (154) has reported striking results in Yugoslavia, where the case mortality from 1919 to 1926 was 20.3 per cent. He observed a fatality rate of 3.4 per cent in 117 antitoxin-treated cases as compared with a rate of 22.3 per cent in 377 untreated cases. Hussameddin (155), Lautier and Dreyfus (156), and others have reported good results in the treatment of severe toxic cases. On the other hand, Stroe and Hortopan (157) and Gabriel (158) were not favorably impressed by the use of serum in toxic cases. Although a few unfavorable reports have been published, it would appear that antitoxin is of value in severe toxic cases and we would advise its use in such patients. The value of the serum in such patients is of course conditioned by a number of circumstances, such as the absence of a concomitant widespread septic state, the earliness of administration of antitoxin, the route of administration, the use of an adequate dose of a potent preparation, etc.

Scarlatinal antitoxin has not proven to be of value in severe septic cases. This, of course, is to be expected, since antitoxin has no antibacterial properties. Weech (159), Benson and MacIver (160), Baxter (161), and Lunin (162) have reported disappointing results. Weech, in particular, observed very fulminating, septic cases with a high mortality in China.

As we have already pointed out, the type of scarlet fever commonly seen in this country at the present time is extremely mild and is commonly referred to as "scarlatina simplex." The mortality is very low, varying from 0.5 to 2 per cent. While most authors are agreed that the early toxic symptoms may be alleviated by antitoxin, nevertheless, as Gordon (163) has pointed out, this early febrile period is not attended by any particular danger. The seriousness of this mild form of disease is largely dependent upon the septic complications such as sinusitis, otitis media, mastoiditis, cervical adenitis, etc., which may occur. We are, therefore, justified in appraising the value of serum in this mild form of the disease largely from a consideration of its value in the prevention of complications. While there is very little *a priori* reason for expecting antitoxin to prevent such complications, and while some authors have presented clinical data showing no reduction in sequelae, nevertheless, the majority are agreed that the complication rate among treated patients is reduced to about one half that in untreated controls.

Craig (164) reported that complications were reduced to about 70 per cent of those occurring in an untreated control group. In Cushing's (165) experience the complication rate was reduced from 45 to 25 per

cent. Veldee, Stevenson, and Mitchell (166) reported a considerable reduction of complications. Hunt (167) reported on the treatment of 2203 patients, of whom 882 received antitoxin, and recorded a similar reduction in complications. Banks (168) has described brilliant results following the use of antitoxin given intravenously in a series of 1204 cases. Only one death occurred, and this was described as not being due to anaphylactic shock. He noted in the average case a fall in temperature by crisis in 6 to 12 hours, the disappearance of faucial edema in about 12 hours, fading of the rash in 12 to 24 hours, and an absence or diminution of desquamation. There was a reduction in the period of hospitalization, and the complication rate was only 4.2 per cent. The author noted a difference in the frequency of immediate serum shock and of thermal reactions among different lots of serum. Thermal reactions and serum disease were common. Banks administered the serum in doses varying from 10 cc. for a small child to 20 cc. for an adult.

In contrast to these reports a number of authors have been very pessimistic in their appraisal of the value of antitoxin. Toomey and Dolch (169) compared a series of 283 treated cases with a group of 60 receiving no antitoxin. They reported a higher complication rate in the treated cases and stated that the serum disease was often more severe than scarlet fever as it occurs at the present time. Toomey (170) in another report of his own experiences, together with a review of the literature, concluded that antitoxin was of no value. Bang and Frederiksen (171), in a review of a large series of cases in the Blegdam Fever Hospital in Denmark, concluded that while antitoxin is of undoubted value in the treatment of properly selected, severe, toxic cases, its use is not warranted as a routine procedure for all patients. They concluded that there was insufficient evidence of a complication-preventing action to compensate for the inconveniences of serum treatment and serum disease to justify the use of antitoxin as a routine procedure. Gordon noted that cardiac complications and nephritis were not appreciably reduced in his cases by serum treatment.

In this connection it is well to recall the experiences of Chandler and Hartshorn (172), which suggest that in some cases the occurrence of serum disease may be a factor in the spread of infection. They reported four patients with scarlet fever treated with antitoxin who developed positive blood cultures for hemolytic streptococci at the time of onset of serum disease. In a later communication these authors (173), together with Burn, reported the occurrence of a similar phenomenon in experimental animals. We, ourselves, have noted the occurrence of a similar phenomenon in a few patients treated with serum. Colebrook (174) suggested that the possibility of the occurrence of this phenomenon

should be considered in the serum treatment of patients with puerperal sepsis.

We feel that antitoxin is definitely limited in its usefulness in the treatment of scarlet fever. We would restrict its use to the treatment of severe toxic cases provided it can be administered early in the course of the disease. The intravenous route as employed by Banks should be seriously considered in severe cases. Otherwise, the intramuscular route should be employed because of the less frequent occurrence of immediate serum reactions. We do not recommend the use of serum in the mild type of disease that is generally prevalent at the present time. The various serious consequences of serum disease outweigh any possible benefit from its use in such cases. There is little or no benefit to be derived from antitoxin in the management of septic types of the disease. Furthermore, antitoxin should not be used in the treatment of septic complications *per se*.

For many years convalescent serum has been used both as a therapeutic agent and for passive immunization in the management of scarlet fever. There is abundant evidence that it is of some value in the treatment of the disease. The results are comparable to those following the use of antitoxin produced in horses. In general, convalescent serum is much less potent than artificially produced antitoxin. For this reason much larger doses need be used, these usually varying from 30 cc. to 100 cc. in the hands of different investigators. The same applies when convalescent serum is used in the rash extinction test. Usually, 0.5 cc. to 1.0 cc. is employed for this purpose, whereas 0.1 cc. of potent antitoxin is generally sufficient.

Convalescent serum has the advantage of being a homologous serum, reactions seldom occur following its use, and patients are not sensitized to a foreign protein. The practical disadvantages of this method consist in collecting and having available the large doses of serum needed for such treatment. Many fever hospitals, however, maintain a serum depot for the express purpose of collecting, processing, and distributing this as well as other convalescent sera.

The Use of Antitoxin in Passive Immunization. Scarlatinal antitoxin has been widely used in fever hospitals for prophylaxis by passive immunization. Doses varying from 5 cc. to 10 cc. (2000 to 4000 units) have generally been employed. According to Cruickshank (175) a 5 cc. dose results in an immunity lasting for 7 to 10 days. Joe and Swyer (176) found that about one third of their cases had relapsed to a positive Dick reaction one week after the administration of this dose. It has been found necessary to use a dose of 10 cc. to secure an immunity lasting for 2 or 3 weeks. Toomey and his associates (177) have reported the occurrence of scarlet fever 6 and 7 days after passive immunization.

While antitoxin has unquestionably been of some value in the prophylaxis of scarlet fever in isolation hospitals, its usefulness is definitely restricted. Relapses occur with annoying frequency following its use. It would appear that in some cases the infectious agent may be harbored throughout the duration of the passive immunity and then result in the development of scarlet fever. Hannah (126) has described the results obtained at the Hospital for Sick Children in Toronto, where every Dick-positive in-patient who has no complication such as asthma, nephritis, or diabetes was passively immunized. We cannot subscribe to a practice of this character. It appears to us that serum disease and future sensitivity to horse serum are a very high price to pay for not completely certain protection against only a very slight chance of developing what, at the present time, is a very mild disease. The low incidence and communicability of the mild disease that is now experienced does not justify passive immunization except under very limited and special circumstances. In any event we would prefer to use, if possible, an adequate dose of convalescent serum for this purpose.

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CHAPTER XXII

OTHER HEMOLYTIC STREPTOCOCCAL INFECTIONS

THE hemolytic streptococcus is one of the most ubiquitous of all microorganisms and probably causes a greater variety of clinical types of disease than any other single bacterial species. To produce such varying types of clinical pictures as a simple, local area of suppuration, an invasive infection of the uterus and neighboring tissues, a rapidly fatal meningitis, and such definite clinical entities as erysipelas and scarlet fever, is, indeed, a remarkable circumstance. Its destructive role as a complicating secondary invader in other conditions is of the greatest importance. Furthermore, it is not surprising that this organism should be blamed as the incitant of a number of conditions such as rheumatic fever and rheumatoid arthritis. Its role in these conditions is correctly regarded as vague and unproven by conservative students of these vexing problems. A huge and confusing literature has developed concerning this organism and the diseases with which it is associated. Out of all this, however, a number of facts have emerged of the greatest importance to the clinician, the epidemiologist, and the immunologist.

There is probably no contribution of greater importance to the general hemolytic streptococcal problem than the development of methods of classification. This has permitted the solution of many epidemiological problems and many obscure clinical inter-relationships. Many attempts had been made in the past to classify streptococci on the basis of fermentation reactions, hemolysis, and other biological responses. The importance of these studies is not to be belittled, nevertheless the most precise classification depends on serological methods. In this latter connection the brilliant studies of Lancefield and of Griffith (see chapter on Scarlet Fever for references) will always be a landmark. For a detailed account of the taxonomy of streptococci, the reader is referred to the recent review of Sherman (1).

Lancefield has divided the streptococci into a number of groups designated by letters A, B, C, etc., through K. Each group is serologically distinguishable from the other on the basis of a group antigen. This antigen, the so-called "C" substance, is carbohydrate in nature. The organisms within each group may be further divided into types on

the basis of the capsular material or "M" substance, which is a protein. Lancefield's results are arrived at through precipitation reactions whereas Griffith employs a slide-agglutination technique. The results obtained by either method are similar in so far as classification is concerned.

The streptococci designated by Lancefield as "Group A" and by Griffith as "Str. pyogenes" includes hemolytic streptococci of human origin and is the one with which we are concerned. This group has been divided into 27 distinct types and undoubtedly more will be added in the future.

Such a classification among hemolytic streptococci is of no less importance than the similar classification of pneumococci. It has provided the foundation for a better understanding of such problems as: (*a*) the limitations of antibacterial serum therapy, (*b*) the tracing of sources of infection, particularly in puerperal sepsis, (*c*) the relationship and identity of strains from scarlet fever and erysipelas with those from other sources, and (*d*) a better understanding of milk-borne epidemics in relation to the clinical features of the cases. These questions will be dealt with in more detail in later sections of this chapter.

Dissociation of Hemolytic Streptococci. The phenomenon of bacterial dissociation is a very important concept in a consideration of the virulence of hemolytic streptococci and in the resistance of the host to infection by that organism. Cowan (2, 3, 4), one of the first to attack this problem, described the occurrence of a "smooth" or virulent type and a "rough" or avirulent type. Todd (5) found that colonies freshly isolated by blood culture had a "matt" appearance. When these were subcultured for a variable period of time, different colonies having a "glossy" appearance were observed. The latter organisms appeared permanently attenuated. Their virulence was not increased by *in vivo* or *in vitro* methods. Todd and Lancefield (6, 7) described a third variant, a so-called matt attenuated variety. They showed that the type-specific substance was related to the matt type of colony. It was also shown that there was a definite relation between passive protection and the type of colony of the organisms used for immunization. An anti-matt serum gave good protection, whereas an anti-glossy serum failed to do so.

Ward and Lyons (8), in their study of streptococcus dissociation in relation to virulence, described a larger series of variants. They frequently obtained on primary isolation from patients a form which they called the "F variant." This phase grew in colonies with an irregular contour and a marked crater-like central depression. The organisms had a small capsule and resisted phagocytosis. This form was generally avirulent for mice. However, if this animal should die, a mucoid or M variant was frequently obtained from the heart's blood. They felt it

likely that the F variant was the parent form of the others. Their second or M variant was heavily capsulated, grew in mucoid colonies, was mouse virulent, and resisted phagocytosis. The attenuated M grew in mucoid colonies but did not resist phagocytosis and was avirulent for mice, although its virulence could be restored by frequent passage. This phase corresponded to Todd's matt attenuated variant. Finally, their C variant corresponded to Todd's glossy avirulent phase.

Dawson and Olmstead (9) had previously described a three-phase system in streptococcus dissociation. They considered the mucoid colony to be the primary or parent variant. Their second phase, growing in a smooth convex colony, probably corresponded to Ward and Lyons' C variant. The end result of dissociation was a very pleomorphic, avirulent form, growing in a smooth flat colony. Dawson, Hobby, and Olmstead (10) have extended their observations to include groups A to G of hemolytic streptococci. They found them all to follow a three-phase dissociation pattern which was designated Mucoid (M), Smooth (S), and Rough (R). Hadley (11), in his recent monograph on microbic dissociation, believed that the three phases of hemolytic streptococcus dissociation as described by Dawson and his co-workers represented the true and significant variation sequences and phases.

The Association of Antigenic Components of Hemolytic Streptococci to Virulence and the Immunity Responses to Them. Bordet (12), in 1897, made the first significant observation concerning resistance to streptococcal infection. He noted that when guinea pigs were injected with streptococci the majority of organisms were quickly phagocyted. Those that remained free developed capsules, resisted phagocytosis, multiplied, and overwhelmed the animals. The resistance to phagocytosis and destruction appeared to depend on the development of a capsule. Hare (13), in 1929, showed that young cultures of a mouse-virulent strain resisted phagocytosis by human blood, whereas older cultures were readily phagocyted. Seastone (14) correlated the resistance of young cultures to phagocytosis with the development of a capsule. A curious discrepancy in this respect is the behavior of the attenuated M variant of Ward and Lyons. This phase, though heavily capsulated, does not resist phagocytosis.

Lyons and Ward (15) showed that virulent streptococci were sensitized for phagocytosis by a type-specific antibody or opsonin. It was only with great difficulty that such antibodies were produced in rabbits. To be successful it was necessary to use living cultures and then not all animals responded satisfactorily. They also found that the blood of a certain number of a group of individuals was capable of phagocytizing and destroying a particular type of organism. This observation was the basis for the development of a technique for the selection of immune

donors for transfusion. This procedure and its practical use will be described in more detail later. It would appear that at least a part and probably the most important part of the recovery mechanism is due to the development of type-specific opsonins. These observations also indicated that the essential antibody for specific sensitization of hemolytic streptococci is one that is capable of uniting with the specific "M" substance of the organism, a point of great importance in a consideration of the development of an immune serum.

A valuable study which clarifies many of the uncertain aspects of the antigenic composition of hemolytic streptococci in relation to immunity has been published by Mudd (16, 17) and his associates. By very special physical methods (sonic vibration, grinding in a dry state at very low temperature, etc.) they were able to break up the bacterial cells and extract from them an antigenic substance which they called labile antigen. This substance is extremely susceptible to oxidation. When it is partly oxidized, it can be restored to activity by a reducing agent. They have obtained the labile antigen in a relatively pure state and find that it has a molecular weight of about 100,000 as determined by ultracentrifugation.

The labile antigen appears to be a molecule which can be split into two parts by acid hydrolysis, a type-specific fraction, and a group-specific fraction. These fractions appear to be identical with the type-specific haptene M and the group-specific haptene C, respectively. They feel that the labile antigen is so oriented in the bacterial cell that the type-specific haptene is reactive (*i.e.*, is probably on the surface) and the group-specific haptene is non-reactive. This labile antigen is antigenic and gives rise to the production of antibodies which promote specific type-agglutination, phagocytosis, and mouse protection against the type of organism from which it was prepared.

The Importance of Classification, Dissociation, and Antigenic Structure in the Production of Antibacterial Streptococcal Serum for Therapy. In the past many attempts were made to produce antibacterial serum for streptococcal infections. These were widely used in such conditions as puerperal sepsis, septicemia, erysipelas, etc. We will not review these various and numerous efforts because it is felt that it can be unequivocally stated that none of these products are of any value. Unfortunately, their use is still persisted in. Not only does a critical clinical appraisal of these sera show them to be valueless, but much experimental evidence has been obtained in support of this. Fothergill and Lium (18) studied the bactericidal properties of three different commercial antistreptococcal sera against a large number of strains of virulent organisms from various sources. In these experiments, varying amounts of the serum and varying numbers of organisms were added to

defibrinated human blood. Thus, leucocytes, complement, and other adjuncts for a bactericidal antibody were present. These sera were found to be entirely ineffective as bactericidal agents. Similarly, Ward and Lyons showed that a number of samples of commercial sera were entirely lacking in specific opsonins for the virulent variant of a large number of strains of hemolytic streptococci.

When we examine this problem further in light of our present knowledge of classification, antigenic structure, etc., our position in condemning these products gains even more support.

We have seen that hemolytic streptococci commonly causing infection in man are divisible into at least twenty-seven different specific serological types. It is also of great importance, as we have emphasized in the chapter on Scarlet Fever and as we shall subsequently emphasize in this chapter, that a particular streptococcal disease entity may be caused by any type. A single type may cause in one individual a septic sore throat, in another erysipelas, in another puerperal scarlet fever, etc. In other words, a specific streptococcal clinical picture is not due to any particular serological type of hemolytic streptococcus. Obviously, therefore, a serum to be useful would have to be type specific for the strain of organism infecting the individual patient. Such a multiplicity of types renders the use of a polyvalent serum unwarranted. Thus, it would be necessary to type the organism in each patient. This procedure would be just as important as the typing of pneumococci in the management of patients with pneumococcic pneumonia. To date, there is no satisfactory method for the rapid typing of hemolytic streptococci, that could be used as a practical procedure in all hospitals. That such will eventually be developed is not beyond hope and expectation.

We have emphasized above that it is extremely difficult to produce a type-specific opsonizing serum in animals. In the past this has been accomplished only by the use of living organisms, and these must be in a virulent mucoid phase. To immunize horses in this manner is out of the question. The supposed antibacterial streptococcal sera that have been produced in the past have undoubtedly been manufactured under circumstances such that one should expect them not to contain specific opsonizing antibody as has been demonstrated for them experimentally. The extreme lability of the important antigen as shown by Mudd and his co-workers offers a reasonable explanation for such difficulty in immunizing animals. Furthermore, with this knowledge and its extension further it is to be hoped that methods will be developed in the future for the satisfactory production of such sera.

We urge, therefore, that these numerous so-called antistreptococcal sera should not be used. Such designations as antisepticemia serum or

antipuerperal sepsis serum should not lead one into a state of hopefulness in the management of such conditions. More harm than good is to be expected from them.

Other Properties of the Hemolytic Streptococcus and the Reaction of the Host to Them. The hemolytic streptococcus elaborates a number of toxic substances, some of which, at least, form a part of its armamentarium. These toxic substances are the erythrogenic toxin, streptolysin, fibrinolysin, leucocidin, and the spreading factor of Duran-Reynals. Unquestionably, the most important of these and the one about which our knowledge is most complete is the erythrogenic toxin. This substance has been dealt with in detail in the section on scarlet fever and will not be considered further at this time. The other substances and the response of the host to them will be dealt with individually.

Streptolysin (Hemolysin) and Antistreptolysin. It has long been known that those organisms classed as *B. hemolytic* streptococci produce a soluble substance (hemolysin) capable of lysing red blood cells. During recent years this substance has been the subject of considerable investigation.

Streptolysin is a relatively unstable substance, being very susceptible to oxidation when it is produced by growing the organisms in a serum-free broth. Todd (19) has developed a method for preparing a reduced streptolysin by the addition of sodium hydroxide and sodium hydrosulfite to the filtrate and carefully adjusting the reaction, which can be preserved in an active state for several weeks. This reduced substance is definitely antigenic and differs in some respects from that produced by growth of the organisms in serum broth. According to Todd (20) serum-free streptolysin is easily adsorbed by filters, is easily oxidized and reduced, is very rapid in its hemolytic activity, and readily combines with its specific antibody. On the other hand, serum streptolysin may have a greater hemolytic activity and is much more stable. It is not easily adsorbed to filters. It combines with its specific antibody, however, only to a small extent. According to Todd (21) both serum-free streptolysin and serum streptolysin are equally effective as antigens for the production of antistreptolysin. In a subsequent paper, however, Todd (22) showed that the streptolysin which was subject to reversible oxidation and reduction was antigenic, whereas another non-antigenic form was produced which was not subject to reversible oxidation and reduction. He showed that streptolysin produced by hemolytic streptococci from human infections was serologically group specific but not type specific. Furthermore, each of Lancefield's groups of hemolytic streptococci produced a different streptolysin.

Todd, Laurent, and Hill (23) have shown that antistreptolysin and erythrogenic antitoxin are independent antibodies, even though scarla-

tinal antitoxin generally contains a high antistreptolysin titer. It was shown that Dick-positive scarlet fever patients may become Dick negative without any rise in antistreptolysin. Moreover, Dick-positive rheumatic fever patients may have unusually high titers of antistreptolysin.

Todd has described details of methods for the titration of streptolysin and antistreptolysin. The minimum hemolytic dose of streptolysin is the smallest amount required to lyse 0.5 cc. of a 5 per cent suspension of rabbit erythrocytes. The unit of antistreptolysin is the smallest amount of serum required to neutralize 2.5 M.H.D. of streptolysin. Todd now expresses this as the reciprocal of the fraction of a cubic centimeter of antistreptolysin which permits expression in whole numbers rather than in fractions.

Numerous clinical studies have been made concerning the relationship of antistreptolysin to various types of infection due to the hemolytic streptococcus. Furthermore, this subject has been explored extensively in a considerable group of diseases thought to be associated in some way with the hemolytic streptococcus such as acute rheumatic fever and rheumatoid arthritis.

The various studies of Longcope (24), Todd, and others indicate that most normal adults possess a definite but low level of antistreptolysin. Values of 100 units or less are generally considered normal. In Longcope's series about 75 per cent had values varying between 25 and 50 units. It is of interest that the age-distribution of antistreptolysin is similar to that with naturally acquired diphtherial and scarlatinal antitoxin. Wilson, Wheeler, and Leask (25) made observations on 16 mothers and their newborn infants. In the majority of cases the mother and infant had a similar titer, in others the infant's titer was higher. The mothers' titers remained at an essentially constant level, whereas the infants' titer diminished rapidly during the first few weeks of life. Among a group of normal subjects of different ages the lowest titers of antistreptolysin were obtained during the age period 3 months to 3 years. In a subsequent paper, Lippard and Wheeler (26) presented further evidence for a direct placental transmission of antistreptolysin.

Numerous investigators [Todd (21), Myers and Keefer (27), Lippard and Johnson (28, 29), Wilson, Wheeler, and Leask (30), Coburn and Pauli (31), Longcope (32), and Stuart (33)] have studied the antistreptolysin response following various hemolytic streptococcal infections as well as that in other conditions thought, by many, to be associated with these organisms. A rise in antistreptolysin occurs following most acute hemolytic streptococcal infections about two to three weeks after the onset. It remains at a high level for a few months and then returns to a normal level. This is a rather curious antibody response. Apparently the level following infection does not persist for as long a time as other antibodies.

It is generally agreed that the antistreptolysin level is significantly higher in rheumatic fever subjects than in normal individuals. Furthermore, a rise in titer usually occurs at the time of onset of rheumatic symptoms. Todd points out that this fact is an argument against the theory that a toxemia is causally related to rheumatic fever. Myers and Keefer found that rheumatoid arthritis was not accompanied by a rise in antistreptolysin titer.

It would appear to be the general consensus of opinion at the present time that a rise in the antistreptolysin level is simply an indication of a preceding hemolytic streptococcal infection. As Longcope, Todd, and others have pointed out, it cannot be taken as evidence to relate causally the hemolytic streptococcus to the disease in question.

The exact function of streptolysin in hemolytic streptococcal disease is not clearly understood. There is no evidence that it plays a particularly important role in such infections. If a large dose is given intravenously to an animal, rapid death occurs, presumably due to intravascular hemolysis. McLeod and McNee (34) have shown that repeated small doses may cause an anemia to develop gradually. However, if streptolysin was an important toxin in this respect one would expect much severer anemias to develop in streptococcal infections than is the case ordinarily. Whether this substance is identical with streptococcal leucocidin is a disputed point. In connection with the role played by streptolysin in the pathogenicity of hemolytic streptococci it is interesting to observe that the dissociated, avirulent, "C" phase of hemolytic streptococci is actively hemolytic. This suggests that streptolysin is not an important offensive weapon of virulent organisms.

Fibrinolysin and Antifibrinolysin. In 1933 Tillett and Garner (35) showed that broth cultures of hemolytic streptococci, as well as the sterile filtrates from such cultures, possessed the property of rapidly liquefying human plasma or fibrin clot. This occurred most rapidly when culture filtrate was added to plasma or fibrin before clotting. Dissolution of plasma clot took place in about 10 minutes. The reaction was much more rapid when a purified fibrinogen clot was employed.

This fibrinolytic property was almost exclusively confined to hemolytic streptococci of human origin. An examination of 38 strains of other species of organisms yielded a negative result. Moreover, only 3 of 18 strains of hemolytic streptococci of animal origin yielded filtrates capable of lysing human clot. In a later communication, Tillett (36) reported the results of a study of a much larger number of strains of hemolytic streptococci with essentially the same result.

In their first communication Tillett and Garner showed that the plasma clot of patients recovered from hemolytic streptococcal infection possessed the property of resisting fibrinolysis. It would appear then

that an antifibrinolytic antibody develops in response to such infections. Methods were described for measuring fibrinolytic and antifibrinolytic activity.

Garner and Tillett (37, 38) succeeded in isolating the fibrinolytic principle in a stable and partially purified form, in which state its properties were further studied. Three methods proved successful: (1) precipitation with alcohol, (2) adsorption on Willstätter's polyaluminum hydroxide B followed by elution with M/10 sodium phosphate buffer at *p*H 7.3, and (3) by vacuum dialysis of the filtrates or the material prepared by adsorption and elution. The two latter methods gave the best results. The active material prepared in this manner was relatively heat resistant, in some experiments withstanding a temperature of 100° C. for 30 to 50 minutes. It gave the usual tests for a protein and was rapidly and completely inactivated by both trypsin and activated papain. The fibrinolytic principle exhibited no hydrolytic action on casein, peptone, or gelatin. It could still be isolated in active form from dissolved human clot. This and other observations suggested to Garner and Tillett that this principle acted like an enzyme or an enzymatic catalyst.

The specificity of this principle is of considerable importance. It acts on human fibrin clot but not on that of the rabbit. Working with purified precursors of fibrin, Garner and Tillett showed that human fibrinogen was readily attacked while rabbit fibrinogen was not, although when rabbit fibrinogen was coagulated with human thrombin, the resulting fibrin was susceptible to fibrinolysin. Neter and Witebsky (39, 40) failed to confirm entirely this specificity. They found that fibrinolysin produced by growing hemolytic streptococci in 2 per cent glucose broth instead of 0.05 per cent glucose broth was active, though feebly so, against both human and animal plasma clot. Moreover, streptococcus viridans, enterococci, and pneumococci produced a fibrinolytic principle with similar properties when cultivated under these conditions. It should be pointed out that growth in 2 per cent glucose broth results in considerable acid production. This may introduce a non-specific effect which might explain the results of Neter and Witebsky. These investigators confirmed the findings of Tillett and Garner when the latter's methods were employed.

It is only natural that, with the discovery of such a substance as fibrinolysin, studies concerning its relationship to infection in man should be conducted. This has been done largely through an investigation of the resistance of fibrin clots to the action of the fibrinolytic substance. Tillett, Edwards, and Garner (41) found that the plasma clots of about 75 per cent of normal individuals were highly susceptible to fibrinolysis. The remaining 25 per cent exhibited varying degrees of resistance. A high degree of resistance occurred following such infections as scarlet fever, erysipelas, and acute tonsillitis. Resistance to

fibrinolysis appeared to develop earlier in patients with suppurative complications. The plasma clot of patients with non-streptococcal infections did not resist fibrinolysis. Lippard and Wheeler made the interesting observation that antifibrinolytic substance was usually present in the newborn infant in much higher concentration than in the mother, and Lippard and Johnson found that children under three years of age lacked the capacity to develop resistance to fibrinolysis. Tillett (42) observed that the fibrin clot of patients dead from hemolytic streptococcal infection was in no instance capable of resisting fibrinolysis. A number of investigators, including Tillett (42), Myers, Keefer, and Holmes (43), Hadfield, Magee, and Perry (44), and Waaler (45), have found that the plasma clot of patients with acute rheumatic fever is antifibrinolytic. Myers and his associates found no rise in antistreptolysin in patients with rheumatoid arthritis.

Thus, it would appear that the development of resistance to the fibrinolytic activity of hemolytic streptococci is a fairly regular phenomenon during or following infection by that organism. It should again be emphasized that the development of this resistance in such a disease as rheumatic fever does not mean that the disease is caused by the hemolytic streptococcus. The exact role played by fibrinolysin in the virulence of the organism is not clear. Nor is it clear what role antifibrinolysin plays in the processes of recovery. In this connection the studies of Hadfield and his associates are of interest in that they showed that "rough" virulent organisms were more active in producing fibrinolysin than "glossy" avirulent cultures. Whether or not fibrinolysin plays a role in inflammatory fixation will be dealt with in our discussion of this phenomenon.

Streptococcus Leucocidin. It has been known for many years that various microorganisms, particularly the staphylococcus, secrete substances that are toxic for leucocytes, causing their death and disintegration. Ruediger (46), in 1905, noticed that filtrates of virulent hemolytic streptococci interfered with the phagocytosis of non-virulent strains. Hektoen (47) found that such filtrates caused definite morphological changes in leucocytes, including loss of motility, swelling of the nucleus, and other changes leading to disintegration. This author found such filtrates to be relatively heat stable. Some of his observations suggested that leucocidin was antigenic. Levaditi (48, 49) suggested that leucocidin was formed and exhibited its destructive effect subsequent to the engulfing of the organism by phagocytes. McLeod (50) noted that actively hemolytic organisms produced leucocidin and believed that there was a definite relationship between the virulence of an organism and its ability to produce a potent leucocidin.

Nakayama (51), in 1920, reported experiments which indicated to him

that leucocidin was a separate substance from hemolysin. Channon and McLeod (52) did not hold this view. They felt that the lysis of red cells and the destruction of leucocytes were manifestations of the same substance. Evans (53) presented evidence that leucocidin was a separate substance from both hemolysin and erythrogenic toxin. Gay and Oram (54) likewise concluded that leucocidin was separable from hemolysin because of its greater thermostability, although it was easily oxidized, and because of its earlier production and greater persistence in broth cultures than is the case with hemolysin. They found that the leucocytes of naturally resistant animals, *i.e.*, dog and guinea pig, were not susceptible to the leucocidin with which they worked. However, the leucocytes of the susceptible rabbit were completely destroyed. They also observed that the clasmatocytes of the rabbit were almost completely resistant to leucocidin, in contrast to the marked susceptibility of the polymorphonuclear cells.

It would appear that leucocidin is a weakly antigenic substance. Nakayama found that antileucocidin could be produced by the injection of culture fluids containing leucocidin. The resulting immune serum neutralized the leucocidin of heterologous strains of hemolytic streptococci as well as that of the homologous strain. Gay and Oram found that antileucocidin could be produced only by the injection of pleural exudates of animals dead of infection. Such exudates contained this substance in greatest amount.

The "Spreading Factor" of Hemolytic Streptococci. In 1929, Duran-Reynals (55) described a property of extracts of various organs, particularly the testicle, which brought about a spread of vaccinia virus when injected into the skin. This spreading factor was active in high dilution and passed through a Berkefeld V candle. He felt that it acted on the tissues of the host and not on the virus. When virus was injected intravenously, it tended to localize in an area of skin previously prepared by the intracutaneous injection of the tissue extract.

In a later paper, Duran-Reynals (56) showed that certain invasive microorganisms, notably the hemolytic streptococcus, contained or produced a soluble substance which markedly increased tissue permeability. This factor was not produced by non-invasive strains of the same organism. He felt that such a factor could pass into the circulation from a local lesion and cause a general increase in tissue permeability which might enhance local infections elsewhere in the body. This spreading factor was entirely non-specific. The factor isolated from streptococci when injected into the skin facilitated the spread of viruses and other unrelated organisms. Dennis and Berberian (57) and Dennis (58, 59) reported experiments in which such a factor appeared to play a role in inflammation. Dennis showed that the activity of the spreading

factor was inhibited by streptococcal immune serum. One of the most recent pronouncements on this subject is that of Pradhan (60), whose experiments suggested to him that the spreading factor was identifiable with the capsular material of the streptococcus when it was free in the surrounding menstruum. He showed that the spreading factor of the hemolytic streptococcus enhanced the power of an avirulent strain to cause an inflammatory lesion in the skin of the rabbit. Pradhan showed that the spreading factor was a separate substance from hemolysin.

Thus, it can be seen that a number of biologically active substances are elaborated by the hemolytic streptococcus. The significance of these substances in the production of disease in man by this organism is not entirely clear. This is particularly so when we consider them in relation to virulence and invasiveness, and in this connection we are inclined to agree with Menkin, Duran-Reynals, and others that the phenomena of virulence and invasiveness should be considered as distinct characteristics of the hemolytic streptococcus. Considering these products from the point of view of invasiveness, we can examine them still further. It would appear that the production of erythrogenic toxin and streptolysin are somewhat fortuitous circumstances. It is to be borne in mind that man is the only animal characteristically susceptible to the erythrogenic toxin. As many investigators have shown, erythrogenic toxin may be produced by completely dissociated avirulent strains of hemolytic streptococci. It has been shown that most of the strains of the Dochez N.Y. 5 streptococcus, a potent toxin-producing organism, are rather completely dissociated at the present time and lack invasiveness in the sense in which we use it. Furthermore, as we have pointed out elsewhere, dissociated avirulent strains generally produce as much and sometimes more streptolysin than freshly isolated, fully virulent organisms.

There is some evidence for believing, on the other hand, that the fibrinolysin, the spreading factor, and leucocidin are more characteristically products of freshly isolated invasive strains, and it is not unlikely that these substances are a definite part of the offensive weapons of this organism. In our discussion of these substances as independent entities, we have indicated certain observations which suggest this association. It should be pointed out, however, that this concept is not sufficiently established at the present time to regard it as a definite biological law. There is need for more intense study of the relationship of the different dissociation phases of the hemolytic streptococcus to the production of these substances. These substances will be considered further in our discussion of the mechanism of inflammatory fixation.

Inflammatory Fixation. The processes involved in the inflammatory fixation of bacteria and other irritants is of great interest not only to the

immunologist and pathologist but to the clinician. By the latter this phenomenon, in certain of its aspects, is being constantly observed. The clinician is constantly being taxed with the problem of a spreading streptococcal infection in the lymphatics, in the skin, in other organs and serous cavities, and finally in those instances where such infections have become generalized. An appreciation of those factors which bring about the prevention of spread of such infections is of immense importance.

The dynamics of inflammatory fixation has been studied by many investigators and by Menkin (61, 62, 63, 64, 65, 66, 67, 68, 69) in particular during the past few years. The reader is referred to Menkin's (70, 71, 72) reviews of his own work, as well as that of others. We can do no more in this treatise than give a brief outline of this phenomenon. When an irritant is injected into the skin, there is first a brief constriction of the neighboring small vessels, followed almost immediately by a marked dilatation. With this there is a marked increase in the permeability of the capillary walls and a flowing of plasma into the tissue spaces. This accounts for the edema in an area of inflammation. The lymphatics in this area become distended with plasma and very soon fibrin webs and thrombi are formed which prevent the further passage of the irritant into the tributary lymphatics. As Menkin has shown, the primary insult would appear to be the actual damage to the capillary wall by the irritant which brings about the increased permeability. Menkin (73, 74, 75) has described the isolation of a crystalline substance, apparently a polypeptide, from inflammatory exudates which would appear to be the active substance responsible for capillary permeability. He has named this substance "leukotaxine." There are other factors involved during the course of inflammation, such as local changes in hydrogen ion concentration, etc. (76), which play a role in the sequences of the process.

Our chief concern here is with the immunological implications of fixation in connection with infection by the hemolytic streptococcus. Menkin has shown that the staphylococcus is rapidly fixed in an area of inflammation as a result of prompt and severe damage to the capillary walls bringing about an early and marked increase in capillary permeability. On the other hand, the fixation of the hemolytic streptococcus is markedly delayed. Menkin has shown, and Dennis and Berberian have confirmed this, that fixation of this organism may not occur for as long as forty-eight hours. Other organisms, such as the pneumococcus, may be intermediate between these extremes. The question which obviously suggests itself is whether or not the various products of the streptococcus which we have described above play a role in the delay of inflammatory fixation of this organism. It would appear from the observations of numerous investigators that the "spreading factor" probably plays a role in this process. On the other hand, it appears

to be a separate substance from Menkin's permeability factor. The spreading factor is relatively thermolabile and non-dialyzable, whereas leukotaxine is thermostable and dialyzable.

Dennis advanced the idea that fibrinolysin may also play a part in delaying inflammatory fixation by the prevention of the formation of fibrin thrombi. It should be pointed out that the activity of fibrinolysin has never been demonstrated *in vivo* but always *in vitro*. Furthermore, as a result of such *in vitro* studies, it is generally agreed that this substance acts on human plasma clot only and not on that of the rabbit. Most of the studies on inflammatory fixation have been made on this animal. Menkin feels that it has not been definitely established that fibrinolysin plays a definite part in the prevention of inflammatory fixation. He feels that the difference in the time of fixation of the staphylococcus and the hemolytic streptococcus is dependent upon the amount of local injury brought about by these respective agents. In conclusion, the part played by fibrinolysin in inflammatory fixation in human infections is in an unsettled state of knowledge.

With this general discussion of the biological properties of the hemolytic streptococci we can pass to a brief discussion of a few of the more important diseases caused by this organism. Before that, however, we wish again to emphasize that no particular serological type of streptococcus can be credited with causing any single disease picture. Each disease picture, whether it be scarlet fever, erysipelas, or a simple peronychia, may be due to any member of that group capable of infecting man. This general concept has been very clearly described by Okell (77, 78, 79) in his Milroy lectures. Each clinical picture is determined by a number of factors such as the portal of entry of infection, the tissue infected, and most important of all, by those properties of the organism which characterize it as virulent and invasive together with the toxic substances secreted and the immunologic reactions of the host to these several biological circumstances.

Septic Sore Throat. Septic sore throat is a disease characterized by a severe angina (pharyngitis and tonsillitis), which may or may not be associated with a scarlatiniform rash. Such complications as cervical adenitis, otitis media, mastoiditis, paranasal sinusitis, etc., are not uncommon. The disease most commonly occurs as a milk-borne epidemic. Septic sore throat is of great interest because many of its obscure epidemiologic and bacteriologic features have been elucidated by the newer knowledge of the classification and dissociation of hemolytic streptococci.

In 1912 Davis and Rosenow (80) and Davis (81) described the results of their investigation of a severe epidemic of this disease in Chicago. They showed that the hemolytic streptococcus isolated from patients

was of high virulence, grew in large mucoid colonies, and was heavily encapsulated. It appeared then that this was a distinct species of hemolytic streptococcus, being characterized chiefly by the large mucoid colonies and the large capsules about the organisms. As a result, epidemic sore throat was regarded for many years as being due to a particular species of organism which was called *Streptococcus epidemicus*.

Walker (82), in 1923, published some observations of unusual interest. He showed that the large moist type of colony, characteristic of *Str. epidemicus*, was a temporary characteristic that might be acquired by other strains of hemolytic streptococci, and that the virulence of a strain that had acquired this colonial character was greatly increased. This was one of the first observations concerning the significance of the mucoid type of colony, which, as we have seen, is so important in the dissociation pattern of hemolytic streptococci. Nearly ten years later Williams and Gurley (83) concluded that the special characters of mucoid colony and capsule formation were not sufficient to describe *Str. epidemicus* as a separate species.

It is now generally agreed that no one variety or type of hemolytic streptococcus is specifically associated with milk-borne sore throat. The large moist colonies simply represent the mucoid phase in the dissociation sequence. With the development of methods of classification it has been possible to trace the origin and evolution of many epidemics of milk-borne sore throat. It has been shown that in the majority of instances such epidemics arise from human origin, that is, streptococcus sore throat or other infection in the milkers or milk handlers. Furthermore, Jones and Little (84, 85) showed that the udders of cows could be infected experimentally with hemolytic streptococci of human origin.

We shall cite several recent epidemics of this disease which illustrate the importance of classification of streptococci in investigating the epidemiology and in showing the relationship between septic sore throat and scarlet fever. Watson (86) described an interesting epidemic which consisted of 135 cases of scarlet fever and 229 cases of sore throat. The distribution of cases casts suspicion on a certain milk supply. A Type 2 strain of hemolytic streptococcus was isolated from the throat of a milker, from the middle ear of the milker's child who had been suffering for over a week from suppurative otitis media, from the teat of a cow which eventually developed mastitis, and from the throats of a number of patients suffering from the disease. While it was not definitely established whether the milk was contaminated directly from the milker or was derived from the cow, nevertheless, all the infections were due to a Type 2 hemolytic streptococcus. This, incidentally, is a common type associated with scarlet fever in England. It is of interest to point out that the ratio of those developing scarlet fever to those with sore throat

is approximately the expected ratio between Dick-positive and Dick-negative reactors among such a population. Under these circumstances it is reasonable to conclude that those with sore throat only were just as likely sources for scarlatinal infection in susceptible contacts as those patients with the typical disease.

Camps and Wood (87) described a similar epidemic of milk-borne septic sore throat in which a proportion of the patients developed scarlet fever. There was a total of about 1600 cases of illness with 6 deaths. The complication rate was particularly high. Bacteriologic and epidemiologic investigation revealed that the infection came from one dairy and from one of three farms supplying milk to this dairy. There were a number of cases of sore throat among the milk handlers on the affected farm. Two children of the head cowman were sick, one with typical scarlet fever, the other with acute tonsillitis only. The wife of this cowman had a sore throat. A Type 2 hemolytic streptococcus was isolated from these various sources. Among patients with illness a Type 2 streptococcus was found in 229 instances out of 250 typed. One cow with mastitis was found to have an organism belonging to the bovine group of hemolytic streptococci. Thus, it appeared quite clear that the original source of infection was of human origin.

These epidemics illustrate further the fact that the development of clinical scarlet fever is, to a large extent, determined by the immunity status of the infected individual. They also emphasize a point we have considered in our chapter on Scarlet Fever. Immunity against this disease protects against certain features only. It does not necessarily protect against the development of a septic throat or other infection by a hemolytic streptococcus capable of causing scarlet fever.

Erysipelas. It is unnecessary to dwell long on a consideration of this particular type of infection of the skin. It was at one time thought that this disease was caused by a specific type of hemolytic streptococcus (*Streptococcus erysipelatis*) which secreted a specific type of exotoxin (erysipelatis toxin) which was different from erythrogenic toxin. We now know these concepts to be erroneous, as we have pointed out above as well as in our chapter on Scarlet Fever. There is no specific type of streptococcus or toxin associated with erysipelas. This is important with respect to treatment of such patients with antitoxin. If antitoxin is to be used, it should be ordinary erythrogenic or scarlatinal antitoxin. Nothing is to be gained by using a product specifically designated as erysipelas antitoxin.

We cannot review all the literature, most of which is inconclusive, concerning the value of antitoxin in the treatment of erysipelas. Its value, if any, is strictly limited. As we have indicated above, a patient with erysipelas may or may not have natural antitoxin immunity. It

is difficult, in the individual case, to decide between these two possibilities. There is no evidence that the typical red appearance of the skin lesion is entirely due to erythrogenic toxin. Attempts have been made to appraise this immunity status by means of a Schultz-Charlton reaction. In our experience this reaction has not yielded clear-cut evidence in erysipelas. At the time when a positive reaction can be read (12 to 18 hours) the lesion has frequently advanced far beyond the area in which the test was done. This area may, at this time, be fading normally.

It is undoubtedly true that some patients might derive some benefit from antitoxin. This would be those patients having no antitoxin immunity and whose illness is characterized by pronounced toxemia. This might explain the favorable results reported by Eley (88) in the antitoxin treatment of infants with erysipelas. Young infants generally have no antitoxin immunity. On the other hand, the majority of older individuals possess antitoxin immunity. Since there is no evidence that such sera possess antibacterial antibody and if it did it would be unlikely to be type specific, there is little justification for its use.

We conclude this section by stating our opinion that, at the moment, there is no so-called antibacterial streptococcal serum whose use is justified in the treatment of erysipelas. Nor is the use of erythrogenic antitoxin justified except in certain selected cases characterized by severe toxemia and in which there is reasonably direct evidence that they lack antitoxin immunity. That antitoxin is not of great importance in the treatment of erysipelas is indicated by the fact that a single individual with adequate antitoxic immunity may have a number of attacks of the disease.

Puerperal Sepsis. In puerperal sepsis one is dealing with an invasive infection of the uterus and its contents and the adnexae which is frequently associated with a localized or generalized peritonitis and is not uncommonly a more widespread bacteriemia. There are few diseases whose obscure epidemiological features have been so profitably cleared up as a result of recently developed methods of streptococcus classification, as puerperal fever.

All cases of puerperal fever are not due to the hemolytic streptococcus. As Colebrook and Hare (89) have shown, many of the milder infections are due to less virulent organisms, such as *B. coli* and non-hemolytic streptococci. On the other hand, the more severe infections are most frequently due to virulent hemolytic streptococci. Colebrook and Hare (90) and White (91) have shown that anaerobic streptococci are fairly frequently isolated during a febrile puerperal state. The former authors isolated such organisms by blood culture from 40 cases in an unselected group of puerperal infections. *Str. pyogenes* was isolated by blood culture from 62 cases in the same general group. These two types of

streptococci, however, were seldom associated together. These organisms are undoubtedly much less virulent than the usual hemolytic streptococcus. In White's study, 50 women were examined during the first stage of labor and 36 per cent were positive for anaerobic streptococci. Among 50 women examined during the last month of pregnancy, 15, or 30 per cent, were positive. Only one instance of febrile illness, and this was relatively mild, occurred during the puerperium in the total group of 100 cases.

Hare (92) showed that about 2 per cent of women have hemolytic streptococci in the genital tract at the time of labor and about 6 per cent during the puerperium, yet it is only rarely that infection of the tissues occurs in women with such positive cultures. Lancefield and Hare (93, 94) showed that the organisms isolated from afebrile women generally do not belong to group A hemolytic streptococci. On the other hand, nearly all strains isolated from severe infections belong to this group. That the strains isolated from the genital tract of afebrile women are of low virulence was indicated by Hare's (95) bactericidal studies. He showed that such strains were easily killed by normal human blood, whereas those from severe infections generally resisted the bactericidal action of normal blood. The blood of afebrile patients with hemolytic streptococci in the cervical secretions was able to kill these organisms in much the same manner as blood from normal individuals.

It can be seen, therefore, that those organisms which are present in the genital tract before or during labor are generally not responsible for the development of severe puerperal infection. The question naturally arises as to what is the source of the severe infections? It is in answer to this that the actual typing of group A hemolytic streptococci has yielded such valuable data.

The most extensive study along these lines has been that of Dora Colebrook (96). In her monograph reporting her own investigations she reviews the work of others in this field. All those interested in this subject should consult this very excellent publication.

These studies show that the source of infection in the vast majority of cases of puerperal sepsis is extra-genital. The most common source is the nasopharynx of some attendant — a doctor, nurse, midwife, etc. An occasional source of infection is the patient's own nasopharynx. There are, of course, various avenues whereby infection may be transferred from the latter source to the genitalia such as fingers, handkerchiefs, etc. Moreover, Hare and Maxted (97) have shown that hemolytic streptococci carried in the nasopharynx may be found in the feces. Fecal contamination of the vulva may be a source of infection by organisms whose primary focus is in the nasopharynx of the patient.

In the past various preparations of antistreptococcal sera have been used in the treatment of puerperal sepsis. We should be vigorous in our condemnation of this practice for the various reasons given elsewhere in this chapter. Leonard Colebrook (98, 99, 100), whose large experience in this field makes his pronouncements authoritative, is unsparing in condemning the use of antistreptococcal sera. Indeed, he has presented good evidence to indicate that serum is harmful. In a group of 40 cases of hemolytic streptococcal puerperal sepsis treated with serum the mortality was 45 per cent. Over the same period of time the mortality was 22.3 per cent in a group of 346 cases due to this organism and treated without serum. Colebrook reviewed the experimental evidence which suggests that the presence of serum disease may be a factor in the actual spread of infection. We have reviewed this evidence in the chapter on Scarlet Fever. This may be a factor of some importance in explaining the higher mortality in the serum-treated group.

The Immunologic Management of Hemolytic Streptoccocal Infections. We have already expressed our opinion concerning the use of so-called antibacterial streptococcal sera. Suffice it again to state that in our opinion there is no such preparation at the present time whose use is justified.

With regard to erythrogenic antitoxin there is very little evidence to show that it is of value in the treatment of septic and invasive streptococcal infections. There is no evidence whatever that it has any effect on the organism *per se*. The development of serum disease following its administration may be associated with the spread of an invasive infection. On the other hand, patients with profound toxemia and an erythematous rash such as severe surgical scarlatina or puerperal scarlatina may derive some temporary relief from some of the toxemic symptoms. The septic aspects of the infection cannot be expected to be benefited.

Numerous attempts (101, 102, 103, 104, 105, 106, 107) were made in the past to treat patients having streptococcal infections with various procedures designated by the term "immunotransfusion." A variety of methods have been described. One procedure has been the injection of a suitable donor with a series of doses of vaccine prepared from the organism derived from the patient. In other instances the donor has been given a single injection of vaccine a few hours before the withdrawal of blood. In some cases the donor, after receiving a course of vaccine, was given an injection of some leucocytosis-promoting substance such as "nuclein" a few hours before the withdrawal of blood. While favorable reports (and these usually on one or a few cases with no statistical significance) have been published, it cannot be said that these procedures are of value, and they have generally been given up except

as a "last-resort procedure" in an occasional case. The most important reason for regarding these measures as ineffective is the fact that it is extremely difficult to produce antibacterial antibody except by immunizing with living, fully virulent organisms. We do not recommend the use of any of these procedures in the treatment of hemolytic streptococcal infection.

Lyons (108) has described a new method for immunotransfusion which, in his hands, has given very good results. Lyons and Ward had shown that young, capsulated organisms in the virulent phase resist phagocytosis in non-immune blood and that phagocytosis is dependent upon sensitization of the organism by type-specific opsonin. They further found that if the blood of a large group of adults is examined, one or more of them might possess specific opsonins for a particular strain. Having such antibody represents an acquired natural immunity probably as a result of an infection sometime previously by that specific type of hemolytic streptococcus. Lyons' method of immunotransfusion was developed on the basis of these findings.

In Lyons' procedure, donors are selected whose serum contains opsonins for the organism infecting the patient. In such a selection it is important that the organism be in a fully virulent phase, and it is generally in this phase when first isolated from the patient. One drop of a sixteen-hour broth culture (usually the primary blood culture from the patient) is inoculated into about 4 cc. of 50 per cent horse serum neopeptone water. This is incubated until the first clouding appears, usually two to four hours, and is then used in the phagocytic test. 0.25 cc. of defibrinated whole blood from the patient is placed into each of a series of small pyrex tubes. A drop of serum from each of a series of prospective donors is added to each tube followed by one drop of the young two- to four-hour culture. The tubes are sealed in a gas-oxygen flame, placed in a rotating box, and incubated at 37° C. for $\frac{1}{2}$ hour. They are then opened, thin smears prepared on clean slides and stained with Wright's stain. These are examined under the oil immersion lens and observation made of the number of organisms contained in twenty-five polymorphonuclear leucocytes and the percentage of leucocytes taking part in the phagocytosis. That individual whose serum promotes active phagocytosis is used as the donor.

It is apparent that this method is dependent on sound immunologic principles. We recommend it in those cases of severe, septic infection if the use of some such procedure is indicated. It should be pointed out, however, that the method has certain drawbacks. It is time consuming. It should be done only by a person experienced in the procedure. It involves considerable labor in that it is frequently necessary to test the serum of a very large group of donors before finding one with the specific antibody for the patient's type of organism. Obviously, of course, all the prospective donors must be of a blood group compatible with the patient.

Chemotherapy in Hemolytic Streptococcal Infections.* The present type of chemotherapy for hemolytic streptococcus infections must be credited to Domagk (109) in Germany. He demonstrated that "Prontosil" given to mice infected with virulent hemolytic streptococci would protect them, while untreated controls routinely died. These results were soon verified by other German and French investigators and many reports appeared of these applications to the treatment of human infections caused by the hemolytic streptococcus.

The earlier studies were conducted using prontosil, which is the hydrochloride of 4 sulfamido-2, 4' diamino azo benzene. A further derivative, prontosil soluble, was produced which is a 2.5 per cent aqueous solution of the disodium salt of 4'-sulfamido-phenyl-2-azo-7-acetylamino-1-hydroxy-naphthalene-3, 6-disulfonic acid. Further work showed that the more simple compound, para-amino-benzene-sulfonamide, was effective in the treatment of hemolytic streptococcal infections.

Meyer-Heine and Huguenin (110) reported on the treatment of 150 cases of erysipelas with prontosil and, with few exceptions, the results were unusually satisfactory. Snodgrass and Anderson (111) reported a series of 312 cases of erysipelas treated with prontosil with uniformly good results. In a series of 38 patients with puerperal sepsis treated with prontosil by Colebrook and Kenney (112) the mortality was 8 per cent compared with mortalities of 23 and 26 per cent in two groups studied before prontosil was used. These same authors (113) reported another series of 26 cases of puerperal infection treated with prontosil without a single death. In a recent report Colebrook and Purdie (114) described a series of 106 cases with a mortality of 7 per cent.

Long and Bliss (115) reported the first group of cases in the American literature treated with prontosil and sulfanilamide. Their report verified the earlier experiments on the curative action in mice infected with the hemolytic streptococcus and included a group of 19 patients with various types of streptococcal infection, including puerperal sepsis, erysipelas, septicemia, scarlet fever, and otitis media with mastoiditis. They concluded that the clinical use of sulfanilamide and its derivatives was warranted in the treatment of streptococcal infections. In a later report by Schwentker *et al.* (116) the value of this drug in the treatment of hemolytic streptococcal meningitis was emphasized. In the symposium on the use of sulfanilamide in the *Journal of Pediatrics* (August, 1937) many favorable reports on the use of these drugs were presented. Flake and Carey (117) described the use of the drug in the treatment of hemolytic streptococcal infections complicating otitis media and mas-

* We are indebted to Dr. Benjamin Carey for assistance in the preparation of this discussion and for the data concerning patients treated at the Children's Hospital, Boston, Mass.

toiditis. Included in this report were four patients with hemolytic streptococcal meningitis complicating mastoiditis, all of whom recovered.

Indications for Sulfanilamide and Prontosil in Hemolytic Streptococcal Infections. ***Septicemia:*** These drugs are definitely indicated in the treatment of this type of infection. No deaths occurred after receiving the drugs in 8 patients at the Children's Hospital, Boston.

Erysipelas and cellulitis: In 20 patients with erysipelas treated at the Children's Hospital, Boston, only 1 death occurred. Fifteen of these patients were under 2 years of age, which is especially significant considering the previous mortality of 30 to 50 per cent from erysipelas in infants. The usual course in these patients was a normal temperature within 18 to 24 hours after receiving the first dose of the drug and disappearance of the skin lesion in 24 to 36 hours.

Meningitis: Five patients with hemolytic streptococcal meningitis at the Children's Hospital, Boston, have been treated with sulfanilamide and prontosil and all recovered. In the 10-year period, 1926–1936, there were 92 patients with this disease and only 1 recovered.

Scarlet fever: The experience of a number of clinicians would indicate that the drug is of no value in the treatment of acute, uncomplicated scarlet fever or in the prevention of complications.

Streptococcus sore throat and cervical adenitis: Results and opinions vary considerably as to the efficacy of sulfanilamide in this type of streptococcal infection. Many patients with throat infections have shown no improvement after receiving the drug and cultures of the throat have remained consistently positive while others have improved very rapidly. Some patients with cervical adenitis have improved rapidly with cessation of fever and complete reduction in the swelling of the glands, while others have shown no change and have required incision and drainage of the infected glands.

Acute otitis media and mastoiditis: Variable and unpredictable results have been observed in this type of infection. Some patients have had cessation of the purulent discharge from the ears within 2 to 4 days after starting the drug and a drop in the temperature to normal within 24 hours. Other patients have shown no improvement. In still others the infection has progressed to involve the mastoid, requiring operation. However, it has been observed that more prompt healing of the mastoid wound takes place when the patient is receiving sulfanilamide, and post-operative care is shortened and simplified.

Streptococcal pneumonia and empyema: If the diagnosis of this type of pneumonia can be made, sulfanilamide should be given. In five patients with streptococcal pneumonia at the Children's Hospital, Boston, a crisis was observed within 24 hours after the drug was started. In three patients with proven streptococcus empyema following pneu-

monia the fluid was absorbed and the X-ray of the lung showed no abnormality after the drug was administered. Two other patients required surgical treatment of the empyema, and no apparent benefit was derived from sulfanilamide treatment.

Idiopathic hemolytic streptococcus peritonitis: Six patients at the Children's Hospital were treated with sulfanilamide in conjunction with incision and drainage. Two of the patients were moribund when treatment was started and died within 12 hours. Two were treated over one week with adequate doses of sulfanilamide before death occurred, but the drug seemed to have no favorable effect. Two patients recovered. In this experience, at least, the drug has been of doubtful value in this type of infection.

Hemolytic streptococcal urinary tract infections: Excellent results have been reported in treating this type of infection. The drug should be given in the usual dosage and continued until repeated cultures of the urine are sterile.

Puerperal infections: We have already reviewed the very excellent results of treatment of this disease by sulfanilamide. It is definitely indicated in this type of infection.

Dosage and Methods of Administration. The dose of sulfanilamide is based on the amount necessary to maintain a blood concentration of 8 to 12 mg. per cent, which is considered to be the optimal level for treatment.

Prontosil soluble (2.5 per cent aqueous solution): One to 1.5 cc. per pound of body weight per 24 hours divided in 4 or 6 doses. The maximum dose for adults is 150 cc. each 24 hours. This preparation is given by intramuscular injection.

Sulfanilamide oral: An initial dose of approximately 0.5 grain per pound of body weight is given with a maximum of 60 grains. The maintenance dose should be 1 to 1.5 grains per pound of body weight per 24 hours divided in 4 or 6 doses. For an adult the maximum dose per 24 hours is 120 grains.

Sulfanilamide parenteral: Sulfanilamide crystals can be dissolved in either sterile physiological salt solution or distilled water at 100° C. to make a concentration of 0.8 per cent. The solution is cooled to 37° C. and preferably given by hypodermoclysis. It may be given intravenously if subcutaneous administration is not feasible. Intrathecal administration of the same solution is possible in meningitis. The initial subcutaneous injection is 100 cc. of the 0.8 per cent solution per 20 pounds of body weight with a maximum initial injection of 500 cc. in adults. The maintenance dose should be about 100 cc. of the 0.8 per cent solution per 40 pounds of body weight every 8 to 12 hours, with a maximum of 1000 cc. each 24 hours in adults.

If sulfanilamide and prontosil are administered simultaneously, the dose of each should be reduced $\frac{1}{3}$ or $\frac{1}{2}$ from the above schedule of dosage.

The full maintenance dose is given for two to three days following clinical improvement and disappearance of organisms from the site of infection. The dose is then reduced about $\frac{1}{2}$ and administration of the drug continued for five to seven days. This plan is followed in order that all organisms may be destroyed and a recurrence of the infection avoided.

Toxic Reactions from Sulfanilamide.

Hematuria: Hematuria was occasionally observed in the patients treated by Colebrook and Kenney. It is undoubtedly extremely rare.

Cyanosis: About 90 per cent of all patients on adequate dosage have varying degrees of cyanosis. It was first thought that this was due to the presence of either sulfhemoglobinemia or methemoglobinemia, but careful studies, including spectroscopic examination and determination of the oxygen capacity of the blood, have shown that the cyanosis was only rarely due to either of these conditions. Proof is lacking as to the exact cause of the cyanosis.

Nausea and vomiting have been observed occasionally in patients receiving these drugs.

Abdominal pain has been reported as occurring in patients receiving sulfanilamide.

Pyrexia: Occasionally patients have been observed to have a sudden rise in temperature while receiving the drug. The temperature did not appear to be associated with the infection for which the patient was receiving treatment, and the temperature returned to normal after the drug was stopped, only to return if the drug was given again.

Lassitude, headache, and mental confusion have been observed in adult patients after receiving the drug. These reactions were temporary and disappeared after the drug was discontinued.

Hemolytic anemia: Harvey and Janeway (118) reported a severe and rapid hemolytic anemia which occurred after sulfanilamide therapy. Transfusion was required to correct the anemia. There seemed to be no doubt that the anemia was due to the drug.

Leucopenia: Leucopenia has been observed in a few patients after receiving sulfanilamide (119). Many others have shown a milder depression of the leucocyte count with suppression of the granulocytes. Frequent leucocyte counts should be done in patients receiving these drugs.

Acidosis: It has been reported that every patient who received sulfanilamide (120) showed some degree of acidosis, as evidenced by chemical examination of the blood. There have been some doubts

about the frequency of this finding; however, clear-cut evidence of acidosis as shown by hyperpnea and by lowering of the carbon dioxide combining power of the blood has been observed in occasional patients. If this condition occurs, the drug should be discontinued and proper steps taken to correct the chemical imbalance.

Rashes: Three types of rashes have been described as occurring after sulfanilamide therapy. A morbilliform eruption similar to that occurring from other drugs is the most common type. An urticarial rash has been observed in two instances at the Children's Hospital, Boston. A type of rash has been described which appeared on parts of the body exposed to the sun in patients who received sulfanilamide, and the suggestion was made that the drug might be a photosensitizing agent. Hageman and Blake (121) suggested that the reaction of fever and morbilliform rash might be compared to the reactions observed in serum sickness.

Optic atrophy: There has been one report of optic atrophy supposedly caused by sulfanilamide. Care should be taken, however, in blaming isolated instances of abnormal findings of this sort as a reaction to the drug.

There is no doubt but that these drugs are extremely valuable agents in the treatment of hemolytic streptococcal infections. However, their use should be restricted to properly selected cases in which the diagnosis is confirmed by bacteriological examination. They should not be used indiscriminately. Furthermore, since an adequate dosage is essential to be effective, and since various abnormal reactions may occur, some of which require emergency treatment, we feel that patients being treated with the drug should preferably be in hospital. Some risk is entailed in using the drug in the home.

Numerous theories have been advanced to explain the mechanism of action of the drug. It is unnecessary to review them here, since, in our opinion, none of them adequately explain the mechanism of action.

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CHAPTER XXIII

STAPHYLOCOCCAL INFECTIONS

THE staphylococcus, principally staphylococcus pyogenes aureus, may be responsible for a great variety of lesions in man. The clinical features of these disturbances cannot be discussed here; nevertheless, for the sake of clarity in our discussion of specific therapy to follow, it will be well to outline the more important groups of diseases caused by this organism.

1. Acute, fulminating staphylococcal sepsis is not uncommon and is almost invariably fatal. In this instance, there is a generalized severe sepsis. Blood cultures are positive and usually exhibit a high bacterial count. In view of the soluble exotoxin to be discussed later, it is of some interest to note that such patients may, at times, present a generalized "scarlatiniform" rash. Stevens (1) has described such cases and we have seen several.

2. Subacute and chronic staphylococcal pyemia is a more protracted type of generalized sepsis characterized by pyemic localizations in various organs and tissues of the body. More commonly, bones, joints, kidneys, serous cavities, lungs, liver, and meninges are involved. The clinical pictures are extremely variable and depend, of course, upon the organ involved and the amount of involvement.

3. Osteomyelitis in its numerous locations and varieties is due to the staphylococcus in about 90 per cent of cases.

4. Acute and chronic pyodermias include a large group of skin diseases such as furunculosis, carbunculosis, sycosis barbae, pustular acne, blepharitis, etc., in which the staphylococcus plays an important role.

There is, of course, a large number of other isolated types of lesions, but the above general grouping is convenient and practical. Moreover, the basic inflammatory type of histopathology is essentially the same in all staphylococcal lesions.

From the point of view of practical specific therapy our chief interest in staphylococcal infections is in connection with the soluble exotoxin of this organism, antitoxin and toxoid.

Staphylococcal Toxin (Historical). The several manifestations of toxic action of filtrates of staphylococcal cultures have been known for many years. The destructive action of such filtrates for leucocytes was

described by Denys and Havet (2), in 1898. This observation was confirmed and extended by Bail (3) and von Lingelsheim (4). Neisser and Wechsberg (5) and Fränkel and Baumann (6) described the hemolytic activity of such filtrates. The rapidly lethal effect was described by Kraus and Přibram (7) in 1906. This property was further studied by Russ (8) and Walbum (9). Neisser and Wechsberg and von Lingelsheim noted the necrotizing effect of staphylococcal filtrates for the skin. Nicolle and Cesari (10) in 1914 and Parker (11) in 1924 observed the same phenomenon. A renewed interest was awakened in the properties of staphylococcal toxin by the Bundaberg disaster (12) in 1928, which has continued to the present time.

Biological Properties of Staphylococcal Toxin. From the above account it is evident that staphylococcal toxin is capable of eliciting several biological reactions.

It exhibits a powerful hemolytic activity for the erythrocytes of a number of species of animals. Rabbits' cells are most susceptible to this action and for this reason are employed in standardization procedures. There is considerable variation in the susceptibility of the cells of different animal species. The hemolytic activity of the toxin is probably not of very great importance in human disease.

Staphylococcal toxin, even when considerably diluted, will cause an area of erythema with marked central necrosis when injected intradermally into rabbits and other animals. This property can be neutralized by antitoxin and has been made use of as a method of standardization.

The so-called leucocidin activity is manifested by a destructive action on leucocytes, as has been shown by the earlier investigators mentioned above, as well as by the more recent studies of Julianelle (13), Pike (14), and Valentine (15). It is likely that this property of staphylococcal toxin is of considerable importance as a noxious agent. Valentine has described a method for the titration of both leucocidin and anti-leucocidin.

The extremely rapid lethal effect of staphylococcal exotoxin is an unusual property for a bacterial poison. Rabbits die within one to three minutes following the intravenous injection of an appropriate dose. Dolman (16) has found other animals, including the guinea pig, horse, cat, and mouse, to be susceptible and subject to this rapidly lethal effect. Russ (8) made a detailed study of the pharmacological action of staphylococcal filtrates and found that in the isolated heart-lung preparation of the cat the toxin had a direct toxic effect on the heart. In addition it appeared to cause damage to the capillaries and smaller vessels of the pulmonary circulation. Dingle, Hoff, Nahum, and Carey (17) have made a detailed study of the action of the heart of rabbits by means of the electrocardiograph following the injection of staphylococcus aureus

toxin. Their studies indicated that the toxin had a direct action on the myocardium, death being due to a failure of the contractile power of the muscle. There was no evidence of a direct action on the nodes or conduction bundle.

In addition to these properties filtrates of staphylococcus aureus may possess the ability to cause oxalated blood to clot in the test tube, as has been shown by Much (18), Gratia (19), Gross (20), and Gengou (21). Menkin and Walston (22) have recently studied the activity of so-called "staphylocoagulase" in inflammation. When injected intradermally in the rabbit, it is absorbed by tributary lymphatics. It fails to provoke a sufficiently powerful local reaction, however, to obstruct lymphatic drainage by the formation of a fibrinous barrier. Menkin and Walston conclude that it probably plays no role in mechanical obstruction to lymph flow. It is of little importance, therefore, in inflammatory fixation.

Certain strains of staphylococci produce a substance that has a powerful toxic action on the gastro-intestinal tract. This substance has been found responsible for a number of outbreaks of so-called "food poisoning." Such outbreaks have been described and others reviewed by Jordan (23, 24, 25, 26) and his associates. As Dolman (27) has shown, this "gastro-enteritis" principle is different from the toxin responsible for the effects enumerated above.

Staphylococcal food poisoning is somewhat different in its clinical manifestations from that due to *B. enteritidis* or *B. aertrycke* infection. In the former instance the incubation period is very short, symptoms appearing in two to four hours after ingesting the contaminated food. There is a violent gastro-enteritis of sudden onset but of relatively short duration, usually not longer than 24 hours. No deaths have been reported to date as a result of staphylococcal food poisoning. Filtrates of broth cultures of staphylococci isolated from suspected food cause this characteristic train of symptoms when ingested by human volunteers. In fact, it is by this method that the etiology of most outbreaks has been demonstrated. The types of food most often involved are those having various kinds of cream or custard filling, such as éclairs, cream pies, layer cakes, etc.

Question of Multiplicity of Staphylococcal Toxins. There is considerable discussion in the literature as to whether the hemolytic, lethal, and dermonecrotic effects of staphylococcal filtrates are due to three different substances or whether these are different effects of a single toxic principle. As determined by absorption experiments, Weld and Gunther (28) concluded that these different effects were due to different substances in the filtrates. Other investigators, however, subscribe to the view that a single toxin is responsible except in the case of the alpha

and beta hemolysins. Dolman found that heating at 60° C. for different lengths of time caused the same relative effects on both the hemolytic and dermonecrotic titers. In studying the action of formalin on staphylococcal toxin Burnet (29) concluded that "all the characteristic activities of the toxin are lost concurrently in the process of formalin detoxication. In none of the experiments with partially detoxicated preparations has there appeared any indication that the three functions of the toxin, hemolytic, skin necrotic and lethal have been dissociated." More recently some evidence has been obtained, as reviewed by Valentine (15), that there may be two hemolytic principles which have been called alpha and beta hemolysin. Since there is practically complete agreement that the lethal, hemolytic, and dermonecrotic action of staphylococcus filtrates are due to a single toxin, the Committee on Standards of the League of Nations (30) has provisionally adopted a method for standardizing staphylococcal toxin and antitoxin depending on hemolytic activity. Separate assays dependent upon the dermonecrotic and lethal effects are not required. It should be pointed out that leucocidin is generally considered to be a separate substance.

All students of the problem seem agreed that staphylococcal toxin is a true bacterial exotoxin and that it is antigenic in so far as its lethal, hemolytic, and dermonecrotic actions are concerned. The immunological reactions of this toxin and its antitoxin are, in general, the same as those which characterize true bacterial exotoxins as a class. Valentine (15) and others, moreover, have published data indicating that leucocidin is antigenic, although Pike (14) published observations to the contrary. Valentine has offered a method for standardizing leucocidin and anti-leucocidin in terms of a standard serum (K serum, B. 8760) issued by the Wellcome Laboratories.

Production of Staphylococcal Toxin. The production of a powerful staphylococcal toxin is dependent upon growing the organism under special cultural conditions, as has been shown by Parker (11), Dolman (16), Burnet (31), Parish and Clark (32), Bigger (33), Bigger, Boland, and O'Meara (34), and Leonard and Holm (35). The most potent toxins are generally obtained by growing the organism in a semi-solid agar with a buffered broth or peptone base. Cultivation is carried out under an increased carbon dioxide tension, the amount varying from 10 to 80 per cent in the hands of different investigators. The toxin is easily destroyed by an alkaline reaction which makes it necessary to use a buffered medium. It is possible that the effect of carbon dioxide may depend upon its buffering action. After 48 hours' incubation the infected semi-solid agar mass is squeezed through cheesecloth and the resulting fluid is centrifuged and filtered through a Berkefeld candle. The reaction of the filtrate is adjusted to slightly acid; *p*H 6.8. Staphy-

lococcal toxin is thermolabile, being destroyed by heating to 60° C. for five minutes.

Standardization of Staphylococcal Toxin and Antitoxin.* Standard antitoxin may be secured in small amounts from the National Institute of Health, Washington, D. C.

The minimal hemolytic dose of the toxin is defined as that amount of toxin which will hemolyze 1.0 cc. of a 2 per cent suspension of rabbit corpuscles in one hour at 37° C.

The proposed International Unit of antitoxin is that amount which will neutralize 200 M.H.D. of toxin.

Standardization of toxin by the hemolytic test. Healthy normal rabbits are bled from the ear vein or by cardiac puncture into 1 per cent sodium citrate in physiological saline. The corpuscles are washed three times with sterile physiological saline, and are made up to a final suspension of 2 per cent of the packed corpuscles. A row of test tubes is set up and each receives 0.5 cc. of increasing dilutions of toxin, 0.5 cc. of physiological saline, and 1 cc. of the 2 per cent corpuscle suspension, making a total volume of 2.0 cc. in each tube. After mixing, the tubes are incubated in a water bath for one hour at 37° C., then left in the ice-box overnight before reading. The end point of the titration is taken as the last tube to show complete hemolysis. Using Dolman's definition of the M.H.D., when 0.5 cc. of the toxin dilution at the end point hemolyzes 2.0 cc. of a 1 per cent rabbit corpuscle suspension, then the hemolytic titer per cubic centimeter of the undiluted toxin is four times the inverse of the toxin dilution in question.

Titration of toxin with the standard antitoxin by the hemolytic test. The standard antitoxin as obtained from Washington contains 20 International units per cubic centimeter. A convenient dilution of this is 1 : 10, so that 1 cc. contains 2 units of the antitoxin. A row of test tubes is set up and each receives 0.5 cc. of increasing dilutions of the toxin. (If the M.H.D. of the toxin has been determined, remembering that 200 M.H.D. are neutralized by 1 unit of the International Standard Antitoxin, the approximate dilutions may be more easily calculated.) 0.5 cc., containing 1 unit of the antitoxin, is then added to each tube.

Finally, 1 cc. of the 2 per cent rabbit corpuscle suspension is added to each tube. After shaking, the tubes are incubated for one hour at 37° C. and placed in the ice-box overnight before reading. Six degrees of hemolysis are read; 3^+ hemolysis has as red a supernatant fluid as the 4^+, but there may be seen a trace of settled intact red cells; 2^+ hemolysis has definite color in the liquid, due to dissolved hemoglobin, but half the original cells are not destroyed and are still intact in the bottom of the tube; 1^+ hemolysis is indicated when most of the cells are in the bottom of the tube, but all of the fluid has a trace of red; in $\pm$ hemolysis practically all the cells are in the bottom, but a water-clear supernatant fluid is present except for a zone of red liquid just above the settled cells. No hemolysis means that all the corpuscles are settled and the entire supernatant fluid is clear. Two plus hemolysis is considered the end point and the amount of undiluted toxin contained in this tube represents the L_h dose of the toxin in question.

* Description prepared from that in the Quarterly Bulletin of the Health Organization of the League of Nations, January, 1935.

Titration of antitoxin with standardized toxin. The collection of blood for the determination of antitoxin content is done under sterile precautions and the serum is separated after the blood has clotted. A row of test tubes is set up, and each receives 0.5 cc. of increasing dilutions of the serum. The tubes are incubated in a water bath at 56° C. for 30 minutes to destroy the complement. 0.5 cc. containing the L_h dose of the standardized toxin is then added to each tube. Finally, 0.1 cc. of the 2 per cent rabbit corpuscle suspension is added to each tube. After shaking, the tubes are incubated for 1 hour at 37° C. and placed in the ice-box overnight before reading. The tube showing 2^+ hemolysis indicates that the dilution of serum contained in that tube contains one International unit of antitoxin. From this the antitoxin content per cubic centimeter may be readily calculated. It has been found that fractions of the L_h dose of the toxin may be used in the titration if the serum has a low antitoxin content without affecting the accuracy of the titration.

Staphylococcal Toxoid. Staphylococcal toxin can be detoxified by formalin with great ease. After only a few hours' incubation in the presence of 0.3 per cent formaldehyde there is a marked reduction in its toxic action. After 48 hours' incubation it is practically completely atoxic. There is abundant evidence to show that such toxoid is an effective immunizing agent in so far as its use is followed, in most instances, by a rise in antitoxin in the serum of the recipient. It should also be pointed out and emphasized that there is no evidence for the existence of more than one antigenic type of staphylococcal toxin, *i.e.*, different strains of staphylococci produce the same antigenic type of toxin. There is, therefore, no point in using a "polyvalent" toxoid for immunizing purposes. Dolman has criticized unfavorable reports on the use of staphylococcal toxoid because it was not a pooled product from several strains. We cannot agree with this criticism for the reasons we have just stated.

Role of Toxin in Staphylococcal Infections. It is difficult to state how important toxin is during the course of staphylococcal infections. There are some observations that indicate that human beings are susceptible to the toxic principle in staphylococcal filtrates. In attempting to immunize patients with subcutaneous injections of small doses of toxin, Weiss (36) noticed that six to twelve hours after such injections there was often local redness and tenderness and occasionally swelling. He reported one instance where the injection of undiluted toxin was followed by intense inflammation and suppuration and with marked systemic symptoms. Pilot and Afremow (37) showed that the intradermal injection of diluted toxin in man caused a zone of erythema with swelling and tenderness within 24 hours. They were able to neutralize these reactions with immune horse and rabbit serum. Stevens (1) reported three cases of acute staphylococcus aureus infection with a scarlatiniform rash. It was difficult to distinguish the rash in these cases from that

in scarlet fever. The rash could be blanched with staphylococcal antitoxin.

It would appear, on the other hand, that there is no correlation between the invasiveness of a particular strain of staphylococcus and its ability to produce a potent toxin. Stevens and Carp (38) studied 31 strains that were obtained from two sources. One group of strains was obtained from the nasopharynx of normal individuals, the other from human lesions, such as furuncles, abscesses, and septicemias. The toxin produced by the various strains was apparently identical, since the reaction of each toxin could be neutralized by the same antitoxic serum. There was no correlation between the amounts and properties of the toxin produced and the clinical origin of the cultures. With respect to the organisms isolated from acute infections, there appeared to be no relationship between the severity of the infection and the ability of the corresponding strain to produce toxin.

Natural Immunity to Staphylococcal Toxin in Health and Disease. The question of natural antitoxin in both healthy individuals and those suffering from various types of staphylococcal infection has been studied by a number of investigators, including Bryce and Burnet (39), Connor and McKie (40), Ramon, Richon, and Descazeaux (41, 42), Murray (43), Blair and Hallman (44), and Dolman (45). In general, the presence of "natural" staphylococcal antitoxin in man is somewhat similar in its distribution to that of diphtheria antitoxin. Umbilical cord blood and the blood of newborn infants contain antitoxin in practically the same amounts as the maternal blood. This is lost during early infancy and is followed by a gradual rise in antitoxin during later years to what is assumed to be a "normal" level, which varies between 0.5 and 1.5 units per cc. of blood. In that group of patients suffering from chronic pyodermias the level is not significantly different from the normal.

The majority of investigators are agreed that the antitoxin level is considerably higher in patients with chronic osteomyelitis. In 59 cases of this disease Dolman found titers of 5 to 6 and occasionally 10 units per cc. Parish, O'Meara, and Clark (46) found the highest titers in patients with deep-seated infections, *e.g.*, of bone, etc. Murray found an average titer of 11.7 in 7 cases of osteomyelitis. He suggests that finding an elevated titer may be of some diagnostic value in obscure bone lesions. Results obtained by Blair and Hallman do not agree entirely with the above. They found normal values in the majority of 80 cases of osteomyelitis. Only one fifth of their cases had three units or more per cc.

Studies of Julianelle and his associates (47, 48, 49, 50, 51, 52) are of interest because they indicate certain immune responses to the bacterial

cell in staphylococcal infections. Two types of specific carbohydrates were isolated from staphylococci. Type A was obtained from strains from pathological sources, while Type B was obtained from saprophytic organisms. They found that skin reactions to the Type A carbohydrate, of the immediate wheal and flare type, occurred in 12 per cent of normal infants and in 65 and 70 per cent of normal adults. This reaction occurred regularly in patients with staphylococcal infections. Skin reactivity was not always associated with demonstrable type-specific precipitins in the serum; the latter being found only in those patients having prolonged or generalized infections. It was found that the bacterial protein caused a species-specific, delayed, inflammatory reaction when injected intracutaneously in hypersensitive individuals.

The recent studies of Lyons (53) on antibacterial immunity in staphylococcal infections are of considerable interest and would appear to offer a new avenue of approach to this perplexing problem. He demonstrated that both toxigenic and non-toxigenic strains were encapsulated in very young cultures grown in infusion broth. Growth in serum inhibited encapsulation. The capsule resisted heating to 100° C. for five minutes but could be removed by mechanical shaking. Toxigenic strains were phagocytized in their non-encapsulated phase. They then reproduced themselves as capsulated organisms and, with the destruction of the leucocyte by leucocidin, were released again into the blood stream. The anticapsular antibody was identified by slide agglutination and appeared to be the effective antibacterial antibody.

From these results it would appear that the A and B carbohydrate antigens of Julianelle and Wieghard are probably group antigens analogous to the group or C carbohydrate of hemolytic streptococci. The capsular antigen of Lyons probably represents a true type-specific substance.

Therapeutic Use of Staphylococcus Toxoid. A number of investigators, particularly Parish, O'Meara, and Clark (46), Murray (43), Ramon, Bocage, Richon, and Mercier (54), Dolman (45, 55), and Connor and McKie (56), treated patients suffering from chronic staphylococcal infections with staphylococcal toxoid. Patients were usually given a series of subcutaneous injections at weekly intervals. The initial dose was small, *i.e.*, 0.05 to 0.1 cc., and was gradually increased at each subsequent injection. A series usually consisted of 4 to 6 injections. In many instances such a series of injections was repeated one or more times. It was found that in the majority of patients there was a gradual rise in antitoxin level to many times the titer at the beginning of treatment. Murray made the interesting observation that there appeared to be an optimum level of antitoxin following the administration of toxoid, and further injections did not increase it. This level is quanti-

tatively similar to that naturally present in most patients with osteomyelitis. It is suggested by Murray that measuring the antitoxin titer in patients' sera provides a reliable index for stopping treatment. There is no purpose in administering additional toxoid when this optimum level has been reached, except, perhaps, for an occasional injection to maintain it.

It is generally agreed that a majority of patients with chronic pyodermias such as recurrent furunculosis, pustular acne, recurrent styes, blepharitis, sycosis barbae, carbunculosis, and other superficial abscesses are improved by treatment with toxoid. It is, of course, difficult to make accurate statistical studies of the value of such procedures because of the virtual impossibility of controlling them properly. One never knows when these infections are going to spontaneously cease or present periodic remissions. Because of the difficulty of making definite objective analyses, many of the favorable reports are not too convincing. Murray's report is an exception. From his tables and the manner in which he studied his cases, it is apparent that many of his patients with chronic skin infections were definitely benefited.

With regard to osteomyelitis there is no convincing evidence that toxoid is of value, although some observers have reported favorable results. Blair and Hallman (57) reported on 38 cases treated with toxoid. An average of 17.9 injections of toxoid were given per patient. This was followed by an increase in antihemolytic titer ranging from 2 to 34 times. They state that "The definite increase in antihemolysin titer was not paralleled by a corresponding improvement in the patient's clinical condition."

In our opinion there is no convincing evidence that staphylococcal toxoid is of any value in infections other than the group of chronic pyodermias. In the present state of our knowledge we cannot advise its use in osteomyelitis and generalized pyemic infections. It should be emphasized also that staphylococcal toxoid is an agent for active immunization only and a considerable period of time must necessarily intervene before the host can derive possible benefit from its injection. For this reason it definitely should not be used in acute staphylococcal infections.

In summary, then, it is our opinion that staphylococcal toxoid is an agent worthy of trial in the treatment of chronic staphylococcal skin infections. For this purpose a toxoid of known antigenic potency should be used. Injections should be given subcutaneously, starting with a relatively small dose, *i.e.*, 0.05 to 0.1 cc. The dose can be increased gradually at subsequent injections. One or two series of 4 to 6 doses each may be given. Five to seven days should elapse between injections. If favorable results are not obtained after such a course of treatment, there is little point in continuing it further. If possible, it is a good

plan to determine the antihemolytic titer of patients' sera before and after such treatment.

Therapeutic Use of Staphylococcal Antitoxin. A great deal of work has been done with staphylococcal antitoxin during the past few years, both in experimental animals and in human infections. Parker and Banzhaf (58) were among the first to produce in horses a staphylococcal antitoxin which was capable of neutralizing the dermonecrotic effect of the toxin.

Burnet (31), in carefully controlled experiments in rabbits, was unable to obtain entirely convincing results in experimental infections with living organisms. When antitoxin was given intravenously immediately after an intravenous injection of living culture, the life of the animals was somewhat prolonged. This author was also able to demonstrate the production of staphylococcus toxin *in vivo*. In three rabbits dying acutely from staphylococcal septicemia the pericardial exudate contained a skin-reacting substance that could be neutralized by antitoxin. When rabbits were actively immunized by the subcutaneous injection of living cultures, they failed to die acutely, but invariably died in from four to thirteen days from generalized pyemic infections. Parish and Clark (32) and Parish, O'Meara, and Clark (46) found that life could be prolonged in rabbits passively immunized with antitoxin.

These experiments have considerable significance. They indicate that antitoxin may protect against acute death in staphylococcal septicemia and suggest that such acute deaths may be due, in part, to the *in vivo* production of toxin. However, it does not protect against late death due to widespread pyemic lesions. This is to be expected, since the antitoxin has no bactericidal action whatever. For certain physiological reasons it is unlikely that antitoxin would be of benefit in subacute and chronic pyemia and osteomyelitis, even if it possessed antibacterial properties. The basic pathology in these conditions is an abscess surrounded by a varying zone of inflammatory tissue. Thus, a barrier, somewhat impermeable to the passage of antibodies, is presented between the regional blood vessels and the actual site of continued infection. Nor can antitoxin be expected to protect against the development of widespread pyemic lesions during the course of acute septicemia. A similar situation exists in other infections. For example, septic complications are not entirely prevented in scarlet fever patients passively immunized with scarlatinal antitoxin.

Several reports of the use of staphylococcus antitoxin in human infections have appeared. Panton, Valentine, and Dix (59) reported favorable results following its use in a small group of patients. Jamieson and Powell (60) were less enthusiastic in their use of antitoxin. While they do not give details of the cases in which antitoxin was used, they summarized their experiences as follows:

"In staphylococcus pyogenic infections it has appeared that staphylococcus antinecrotizing serum has produced no uniformly good results. Vigorous intravenous and intramuscular antitoxin treatment in such cases extending over periods of from one or two to thirty days has sometimes had a favorable effect in reducing temperature and preventing daily chills, but blood cultures have in the main continued to be positive during serum treatment, and metastatic infections have not been decreased or prevented. Local applications of staphylococcus antitoxin to abscesses have had a favorable effect in some cases; however, the efficacy of such treatment has not exceeded that commonly reported in the use of bacterial lysates." It is of interest to note the similarity of these results in human beings to those discussed above in experimental animals.

Dolman (61) has reported a large series of various types of staphylococcal infections and is an advocate of this form of therapy. It is difficult for the reader to make an independent appraisal of the results. His condensed mortality table is not particularly convincing. Enormous doses of antitoxin over a prolonged period of time were used in many of his cases.

In summary, our own opinion regarding the use of staphylococcus antitoxin is that it may relieve certain symptoms and may, in some cases of acute septicemia, prevent early death. There is little reason to suppose it to have much curative value in subacute or chronic pyemia and osteomyelitis. Indeed, it might be said that antitoxin might change an acute, fulminating septicemia into the chronic type of pyemia with continuing positive blood cultures. In the latter type of disease there is little to be expected from antitoxin treatment on the basis of existing evidence. It should be emphasized again that this antitoxin has no demonstrable bactericidal effect. We feel, therefore, that the therapeutic usefulness of staphylococcal antitoxin is very limited. There is accumulating evidence to suggest, however, that a potent anti-leucocidin may be of distinct value. Such observations as those of Valentine and of Lyons indicate definite hopefulness for therapeutic developments along this line.

If antitoxin is used, it preferably should be given in moderate doses intravenously. We fail to see any purpose in giving enormously large doses over prolonged periods of time.

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CHAPTER XXIV

MENINGITIS

MENINGITIS is an infection involving the meninges of the brain and spinal cord and the subarachnoid spaces. This inflammatory process may be due to a considerable number of pathogenic agents. Moreover, the subarachnoid cavity is, in a sense, a peculiarly defenseless anatomic region as far as the general immunity processes of the body are concerned. It is for this reason and because of certain anatomic and physiologic situations making the development of serious complications likely, and because of damage to vital tissues, that meningitis is such a fatal disease.

It is not in the province of this text to discuss the clinical features of meningitis nor the clinical diagnosis. Suffice it to say that with certain rare exceptions there are no clinical features by which the bacteriological type can be recognized. In general, of course, meningitis in an individual presenting numerous petechial skin lesions is most frequently due to the meningococcus. On the other hand, absence of skin lesions does not negative this diagnosis.

Certain organisms are more common causes of meningitis than others. The most frequent causative agents are the tubercle bacillus, the meningococcus, the influenza bacillus, the hemolytic streptococcus, and the pneumococcus. The following table describes the relative frequency of the common bacteriological types admitted to the Children's Hospital in Boston, over a period of sixteen years. These figures are taken from those published by Fothergill and Sweet (1) and Fothergill (2).

TABLE I

THE RELATIVE FREQUENCY OF THE MORE COMMON BACTERIOLOGICAL TYPES OF MENINGITIS IN CHILDREN

Causative Organisms	*No. of Cases*
Tubercle bacillus	321
Meningococcus	187
H. influenzae	129
Hemolytic streptococcus	105
Pneumococcus	107
All others	77
Total	926

There was some change in the distribution of the different types during the last four years of this survey. The following table describes this change:

TABLE II

THE FREQUENCY OF THE DIFFERENT TYPES OF MENINGITIS ADMITTED TO THE CHILDREN'S HOSPITAL, BOSTON, DURING THE YEARS 1933 TO 1936

Causative Organisms	*No. of Cases*
H. influenzae	51
Pneumococcus	38
Hemolytic streptococcus	36
Tubercle bacillus	31
Meningococcus	27
All others	38
Total	221

Taken from Fothergill (2).

It can be seen that tuberculous meningitis has become relatively less common than in previous years. *Hemophilus influenzae* was the most frequent causative agent during this later four-year period. These figures, of course, represent those cases seen in a children's hospital only. Moreover, this analysis represents the distribution of types during a non-epidemic period. At no time during this survey was there an epidemic increase of meningococcic meningitis. Other organisms, of course, such as staphylococci, colon bacilli, etc., occasionally cause meningitis. Rivers and Scott (3) have described a filterable virus as a cause of rare cases of acute lymphocytic meningitis.

Bacteriological Diagnosis of Meningitis. An examination of the above figures indicates that a proper diagnosis of meningitis is dependent upon bacteriological procedures. If these are carefully done, diagnosis can be made in most instances within a relatively short period of time. There are two essential procedures which should be carried out in every case. The first of these is the examination of carefully prepared smears of the cerebrospinal fluid stained by Gram's method, and the second is to culture the fluid on a proper medium.

We shall briefly describe the useful bacteriological procedures in each instance. In tuberculous meningitis a smear of the cerebrospinal fluid is generally prepared after some method of sedimentation. One method is to allow the fluid to stand for a few hours, during which a fine fibrin pellicle or web will form. This pellicle with the various cellular constituents of the spinal fluid enmeshed within it can be floated upon a clean slide, dried, fixed, stained by the Ziehl-Neelsen technique, and examined for tubercle bacilli. Another method is to place a cover slip in the bottom of a small glass and pour a few cubic centimeters of the spinal fluid over it. Most of the cellular content of the fluid will have settled on the

cover slip in a few hours, after which it can be removed, dried, and stained in the usual manner. The latter method has been very satisfactory in our experience. Guinea pig inoculation can and should be employed, but diagnosis by this method is, of course, considerably delayed.

In all cases of acute purulent meningitis the cerebrospinal fluid should be smeared directly on a slide, stained by Gram's technique, and examined. In meningococcus meningitis the diagnostic finding is the demonstration of morphologically typical Gram-negative diplococci. In many instances meningococci are present in very small numbers and considerable search is often necessary to find them. In other cases, however, they may be found in large numbers. The impression is given in many textbooks of medicine that it is necessary to find such organisms in an intracellular location to make a diagnosis. Such a concept is incorrect. The organisms are usually found extracellularly in early cases where there has been no immune response on the part of the patient. It makes no difference, in respect to the immediate therapeutic course to be followed, whether the organisms are intracellular or extracellular, providing they are morphologically typical. Rake (4) and Maegraith (5) have reported making rapid diagnoses by means of a precipitation test with supernatant cerebrospinal fluid and monovalent type-specific immune serum. This procedure offers a rapid method of diagnosis in many cases. The reaction probably depends upon the presence of soluble specific polysaccharide in the cerebrospinal fluid.

The influenza bacillus is one of the most easily recognized of the common organisms causing meningitis, yet its presence is frequently overlooked. In its normal state this organism is an extremely small, Gram-negative bacillus. In the cerebrospinal fluid, however, it exhibits a marked pleomorphism which is pathognomonic of it. A great variety of morphological forms may be seen, including small bacilli, short diplobacilli which are often confused with the meningococcus, larger bacilli, and long filamentous forms. In the majority of cases, moreover, the organism is present in very large numbers. In most cases a rapid diagnosis can be made or confirmed by a precipitation test with the supernatant cerebrospinal fluid and a properly prepared immune serum (2).

One of the most characteristic features of the spinal fluid in pneumococcal meningitis in most cases is the fact that organisms are present in extremely large numbers. Indeed, the cloudiness of such fluids is often due to the large number of organisms rather than to a high leucocyte count. Under these circumstances, it is possible to centrifuge the fluid for a few minutes at low speed to sediment the leucocytes and then proceed with diagnostic procedures on the supernatant fluid which provides a satisfactory suspension of the organisms. A bile solubility

test can be done on a portion of it. One of the rapid pneumococcal typing procedures such as the Neufeld or Sabin technique can be applied directly. Under such circumstances it is possible not only to make a diagnosis of pneumococcus but also of the type within a short time after a lumbar puncture. It should be pointed out that the Type III Pneumococcus is frequently present in long chains and may be confused with the hemolytic streptococcus. However, even with the Gram stain it is often possible to see the characteristically large capsule of this particular type.

The morphology of the hemolytic streptococcus is usually characteristic, although it is occasionally confused with the pneumococcus, particularly when it occurs as diplococci and in short chains. Diagnosis under the latter circumstances will depend upon the exclusion of the pneumococcus by the procedures described above and by cultural methods.

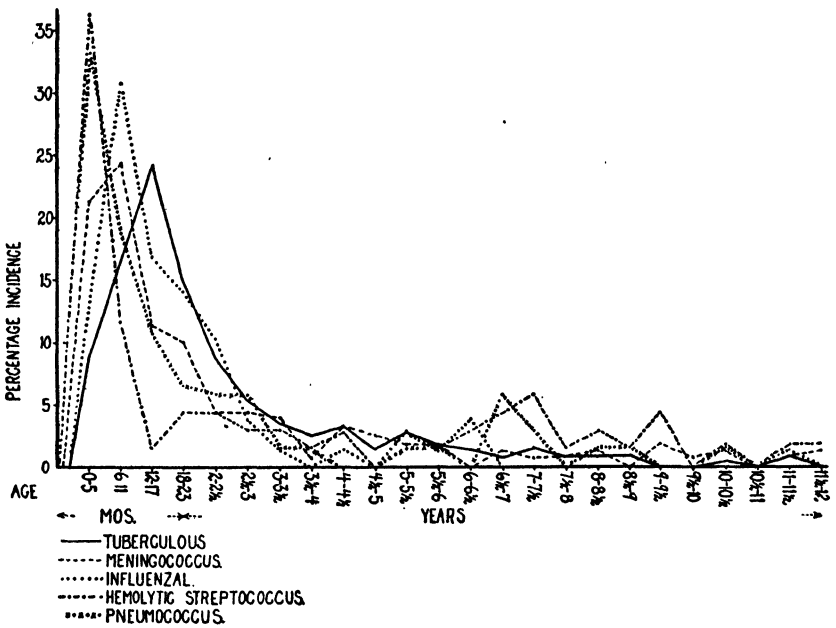
In considering the cultural diagnosis of meningitis, we shall confine ourselves to the organisms producing acute purulent disease. The tubercle bacillus presents a rather special problem and is generally recognized by the procedures we have already outlined. From the figures which we have given indicating that one of several different organisms can be expected in a cloudy spinal fluid, it is obvious that some cultural procedure should be employed that will be suitable for the prompt growth of any one of them. In our experience we have found the following procedure to be the most satisfactory: At the time of diagnostic lumbar puncture, a small quantity of spinal fluid is allowed to flow onto each of two chocolate agar slants. One of these is incubated aerobically and the second is incubated under an increased carbon dioxide tension. A very useful and practical method for securing this is to inoculate heavily a plain agar slant with *Bacillus subtilis*. With the cotton stoppers in place, this tube is joined to the chocolate agar slant by means of a short section of heavy rubber tubing, thus making a closed system. The meningococcus is a microaerophilic organism and grows luxuriantly and rapidly under these conditions. We have frequently encountered excellent growth in this manner, whereas no growth occurred in the tube incubated under ordinary aerobic conditions. Chocolate agar is ideally suited for the growth of the influenza bacillus because it provides both the X and V growth factors. Moreover, the hemolytic streptococcus, the pneumococcus, and other organisms commonly causing acute purulent meningitis grow very well on it. The influenza bacillus will not grow on such media as ascitic or tryp agar, which are commonly employed for culturing the meningococcus. We have seen many instances of failure to make a bacteriological diagnosis of meningitis because of using an unsuitable culture technique.

Pathogenesis of Meningitis. It is generally agreed that the meningococcus enters the body by way of the nasopharynx, having been carried there as a droplet infection from a case or carrier. Numerous studies have been made of the distribution of carriers during the course of epidemiological studies of the disease, particularly during the World War. It was found that carrier rates increased during the winter and spring months and under conditions of marked crowding of people. For the latter reason meningococcus meningitis is often regarded as a military disease. Glover (6) made numerous studies of carriers in army barracks and showed that such a simple procedure as greater spacing of beds lessened the incidence of infection. It should be pointed out, however, that an increased incidence of the disease does not always parallel a rise in the carrier rate. Norton and Baisley (7) found no correlation between crowding in homes and increase in the carrier rate during the Detroit epidemic of 1928–29. Rake (8) showed that a carrier rate could be quite high among a group of individuals without the disease occurring. Dudley and Brennan (9) made a similar observation. They found a greater incidence of meningitis during a period when the carrier rate was low than during a later period with an exceedingly high carrier rate.

Studies of the distribution of carriers during an epidemic of the disease are not necessarily of significant value because of the fact that we have no simple test for determining susceptibility of the individual to infection nor for ascertaining the virulence of the organism. Both of these qualities can be determined by rather elaborate procedures which are not applicable to a large-scale study. A few observations of this character which have been made, particularly by Rake (8), would indicate that a large percentage of carrier strains are probably avirulent.

There has been considerable discussion concerning the route by which meningococci reach the meninges from the nasopharynx. One school of thought proposed by Netter and Debré (10), in 1911, is that the organisms enter the subarachnoid space as a result of direct extension through the cribriform plate. According to this view, organisms are transported in the perineural lymphatics and spaces of the olfactory nerve. The second view, supported by Westenhoeffer (11), Elser (12), Herrick (13), and others, regards the organisms as first invading the blood stream and then localizing in the meninges. It has been demonstrated frequently that blood cultures may be positive early in the disease and become negative later. This has been our own experience. In fact, the bacteriemic stage is often of very short duration in most cases. Organisms were cultured from the petechial skin lesions by Muir (14) in 1919. McLean and Caffey (15) made smears of the blood obtained by pricking such skin lesions and were able to demonstrate Gram-negative diplococci in a large percentage of their cases.

It would seem strange that there is not a much greater frequency of meningitis due to other pathogenic and invasive organisms that are frequently present in the nasopharynx if such agents were carried to the meninges by the perineural lymphatics of the olfactory nerve. If this mode of transport is correct, we can legitimately wonder why we do not have a great deal more hemolytic streptococcal meningitis, for example. It would appear more plausible that if organisms are carried from the nasopharynx by these lymphatics, they are emptied into the venous channels of the cranial cavity, which would amount, in effect, to an intravenous inoculation. In our discussion of immunity in meningitis we will describe additional indirect evidence in support of the hematogenous route of infection. The studies of Fothergill and Wright (16), in particular, of immunity in *H. influenzae* meningitis supports this theory. In our opinion so-called primary meningitis is a hematogenous infection of the meninges. It is true, of course, that the subarachnoid cavity may be secondarily infected by direct extension of infection in regional structures such as in otitis media, mastoiditis, ethmoiditis, and sphenoidal sinusitis. Meningitis is a not uncommon complication following fracture of the skull, particularly fractures of the base. In these instances the



COMPOSITE CHART OF THE RELATION OF AGE TO INCIDENCE IN THE FIVE COMMON TYPES OF MENINGITIS

Taken from Fothergill and Sweet (1).

disease occurs by direct extension of infection. These various instances of secondary meningitis are more frequently due to the hemolytic streptococcus and the pneumococcus.

Age Distribution and Immunity in Meningitis. Meningitis is primarily a disease of early life. The above figure describes the age distribution of the more common types during the first twelve years of life. It is obvious that the majority of cases occur from a few weeks after birth until three to four years of age. This is of general immunological interest because it is during this period of life that an individual is extremely susceptible to all types of infection. As age advances it is quite likely that many individuals develop some immunity against meningococcal infection. That the blood of many individuals possesses bactericidal properties has been demonstrated by a number of investigators, including Davis (17), McKenzie and Martin (18), Matsunami and Kolmer (19), Matsunami (20), Heist, Solis-Cohen, and Solis-Cohen (21), and Silverthorne and Fraser (22). This bactericidal power should provide protection against the development of the disease in those individuals possessing it if the hematogenous route of infection is the correct explanation. Furthermore, this would explain the low incidence of the disease even during an epidemic. We shall discuss this feature again when we consider *H. influenzae* meningitis.

The Types and Antigenic Structure of Meningococci. It was recognized early in the course of investigations of the meningococcus by Dopter (23), Arkwright (24), Trautmann and Fromme (25), Elser and Huntoon (26), and others that these organisms could be divided into at least two main serologic types with some overlapping between them. These two groups were variously designated as Types I and II, Types A and B, and meningococcus and parameningococcus. In 1915 Gordon and Murray (27) further divided the meningococci into four types by absorption of agglutinins. Nicolle, Debains, and Jouan (28) described two additional types which they designated as C and D. Types I and III of Gordon and Murray correspond to Type A of the French and the "meningococcus" of Dopter, while their Types II and IV correspond to Type B of the French and Dopter's "parameningococcus." All are agreed that there is much antigenic overlapping among these types. Moreover, a given strain may gradually change with regard to its dominant type characteristic. For example, Gordon had preserved his original Type I strain in a dried state. Other subcultures have been maintained by cultivation for over twenty years. These latter cultures appear to be Type III. Maegraith (29) and Branham (30) have studied living transplants of the original dried material and found it to be Type I. This antigenic change and overlapping is best explained by regarding the meningococcus as being composed of four antigenic

components. The types will depend upon which of these is dominant at the time.

During recent years these type differences have become less clearly definable. It is extremely difficult or impossible to differentiate between Types I and III. Branham (30) suggests that meningococci be considered as falling into three types to be designated as Types I–III, II, and IV.

During the past few years our knowledge of the antigenic structure of the meningococcus has been increased through the application of chemical methods by a number of investigators. Zozaya (31) and Zozaya and Wood (32) described the presence of polysaccharide and nucleoprotein components in these organisms. Rake and Scherp (33, 34, 35) reported the demonstration of a type-specific polysaccharide in Type I meningococci. This substance was the sodium salt of a polysaccharide acid which was composed of a nitrogen-containing sugar and a phosphoric acid unit. The phosphorus was found to be firmly held in an organic combination. This substance was reactive in a dilution of 1 : 8,000,000 with a Type I monovalent serum. In addition, these organisms contain a group-specific carbohydrate common to all types of meningococci as well as to other related organisms. Rake and Scherp have shown that the type-specific polysaccharides of Types I and III meningococci are apparently identical. A different specific carbohydrate was isolated from Type II organisms.

The Toxins of Meningococci. Numerous workers have been concerned with the properties of the endotoxin of the meningococcus. This problem was studied in great detail by Gordon (36), Murray (37), Malcolm and White (38), and others. This substance can be obtained by extraction of the bacteria by a variety of methods. Moreover, the meningococcus is extremely susceptible to spontaneous autolysis with resulting liberation of the endotoxin. Meningococcal endotoxin will cause death in rabbits, guinea pigs, and mice and will produce a skin reaction when injected intradermally. An anti-endotoxin can be prepared which is usually not very potent and which does not neutralize according to the laws of multiple proportions.

Ferry and his associates (39, 40, 41, 42, 43, 44, 45) described the production of a soluble exotoxin by the meningococcus. They secured their substance by growing the organisms on the surface of hormone broth for four days. They described the production of a specific exotoxin by each of the four Gordon types of meningococci as well as a group-specific exotoxin produced by all types. Ferry and his co-workers immunized horses by injection of this material and claimed to have produced a true antitoxin. We feel that this so-called exotoxin is no different from the endotoxin as it had always been known. The obser-

vations of Malcolm and White (38), Maegraith (46), and Petrie (47) do not substantiate Ferry's claims. Kirkbride and Cohen (48) compared antisera produced by injection of living cells and broth filtrates and were unable to demonstrate qualitative differences between antitoxic and antibacterial sera. They found, in fact, that their antibacterial serum was more potent in protecting mice against virulent living cultures.

In summary, we feel that the therapeutic activity of so-called meningococcus antitoxin is due to the presence of antibacterial and antiendotoxic antibodies. While Ferry's serum undoubtedly has therapeutic value (49, 50, 51), its use, on the assumption that it is an antitoxin, is not recommended.

The Production of Meningococcal Antiserum. Various methods have been employed from time to time for the production of antiserum. In 1908, Flexner and Jobling (52) used alternate subcutaneous injections of living organisms and an autolysate. This method was satisfactory but required ten to twelve months to produce a potent antiserum. Later, Amoss and Wollstein (53) described a more rapid method for producing a polyvalent serum which consisted in giving three intravenous injections of living organisms at daily intervals to be followed by eight days of rest before repeating the series. The essentials of this method are being followed in many laboratories at the present time, the differences in procedure being largely due to variation in the number of strains used for immunization. Wadsworth and Kirkbride (54) favor the use of from four to six carefully selected strains. Other workers have employed a larger number.

Standardization of Meningococcal Antiserum. The standardization of antimeningococcal serum is still in a very unsatisfactory state. We have no standard unit of reference for making comparative titrations. A number of immunologic reactions have been employed for determining the relative antibody content of different sera. Flexner and Jobling (52) and Nicolle, Debains, and Jouan (28) used the complement-fixation test. Amoss and Wollstein (53) determined the agglutinin content, and Zozaya (55) and Mishulow (56) employed precipitation tests. At the present time in this country, sera are compared by the agglutination reaction with a standard serum provided by the National Institute of Health.

In 1935 Miller (57) made use of the observation of Nungester, Wolf, and Jourdonais (58) that animals could be infected by injecting organisms suspended in mucin and showed that mice could be infected with meningococci suspended in this agent. Since then a number of investigators, including Branham (59), Mishulow, Melman, and Sklarsky (60), and Rake (61, 62), have attempted the standardization of antimeningococcal serum by a mouse-protective test. It is hoped that in the future some standard method will be developed along this line. A test which

determines the capacity of a serum to protect an animal against a virulent infection is obviously a better measure of bactericidal antibody content than an immunologic procedure such as the agglutination reaction.

The Treatment of Patients with Antimeningococcal Serum. Various methods have been recommended from time to time for the treatment of patients. The methods have usually differed mainly in the route of administration of serum. In our experience good results have been obtained by the intrathecal administration of serum in doses of 10 cc. for a small child and 15 to 20 cc. for an adult, three doses being given at twelve-hour intervals to be followed by a daily injection as long as indicated. Patients who are seen early and who have petechial skin lesions or other evidence of bacteriemia should be given at least one dose (30 to 60 cc.) of serum intravenously.

If the serum is effective, evidence of benefit is usually observed within two to four days of its administration. Such evidence consists in a general improvement in the clinical condition of the patient, a gradual fall in temperature, fall in the leucocyte count of the cerebrospinal fluid, a return of sugar to a normal level in the spinal fluid, and the occurrence of negative cultures. Special therapeutic procedures may be necessary for the management of various complications, such as block, should they occur. For a detailed account of the treatment of meningococcus meningitis and its complications, the reader is referred to the monograph by Blackfan (63).

Recently emphasis has been placed on the intravenous administration of serum. Hoyne (50) has reported excellent results following the injection of serum by this route only. Many clinicians are of the opinion that the inflammatory reaction in the meninges caused by the intrathecal injection of serum is harmful. While it is perfectly true that intrathecal serum administration does cause an increased inflammatory reaction as evidenced by a temporary increase in the cell count of the spinal fluid, we are not convinced that this is a seriously harmful circumstance. In an antibacterial mechanism, it is essential for antibody, in adequate concentration, to come into immediate contact with organisms. Numerous investigators (64, 65, 66, 67, 68, 69, 70, 71, 72) have demonstrated that the meningovascular barrier is relatively impermeable to antibodies, although permeability is undoubtedly increased under the influence of inflammation. It is questionable, nevertheless, if an effective amount of antibody enters the subarachnoid cavity following its intravenous administration. In our opinion intravenous serum should certainly be given to those patients in whom there is reason to expect the presence of bacteriemia. Infection localized in the subarachnoid cavity should be treated by the local injection of serum.

There are many factors which influence the results obtained by serum

therapy in meningitis. The disease is especially serious in young infants who are prone to develop serious complications such as hydrocephalus. An extremely important factor is the time of administration of serum. There are few diseases where it is more important to make an early diagnosis. The following figures taken from McKhann (73) illustrate the influence of late diagnosis on mortality.

CASE FATALITY BY AGE GROUPS, IN RELATION TO DURATION OF THE DISEASE BEFORE TREATMENT

AGE GROUP	DURATION OF DISEASE BEFORE TREATMENT	CASES	DEATHS
Under 1 yr.	Under 1 wk.	16	4
	1–2 wks.	4	3
	2–4 wks.	10	9
	4–8 wks.	7	7
	Over 8 wks.	2	2
1–2 yrs.	Under 1 wk.	4	1 (fulminating)
	1–2 wks.	0	0
	2–4 wks.	2	0
	4–8 wks.	3	1
	Over 8 wks.	1	1
2–12 yrs.	Under 1 wk.	19	4 (fulminating)
	1–2 wks.	1	0
	2–4 wks.	4	2
	Over 4 wks.	1	0

Taken from McKhann (73).

It is extremely important to be sure that one is using a serum that is effective against the particular strain infecting the patient under treatment. Part of the management of patients should consist in culturing the organism from the cerebrospinal fluid and ascertaining if it is agglutinated by the serum in use. If not, an effort should be made to find another lot of serum to replace it. As we have pointed out above, the agglutination reaction is not a certain criterion of therapeutic effectiveness of serum; nevertheless, when employed in this manner, it may give valuable information in guiding one to a more effective antiserum.

Much has been written in recent years expressing some doubt as to the value of serum treatment in meningococcus meningitis. It is pointed out, for example, that before serum was available mortality rates varied from 40 per cent to 80 per cent in different epidemics. It is also claimed that mortality rates of this order of magnitude are being observed at the present time in serum-treated patients. Those who criticize the value of this form of therapy point out that since the advent of serum all

patients, if diagnosed, are usually treated. We, therefore, have no untreated series as controls.

While having due regard for these criticisms, we feel that there is little question but that treatment of early cases with a properly selected serum has caused a marked reduction of mortality. The strikingly rapid clinical improvement observed in many cases is quite at variance with what one might expect in untreated patients. It is perfectly true, however, that the general mortality in many series of treated cases is still about 30 per cent or more, a figure no better than that reported many years ago by Flexner (74) when he first undertook the production of antimeningococcus serum.

Cormack (75) has published a valuable paper which should answer, in part at least, the objections of those who point out that we have no controls of serum therapy. This investigator treated a large number of cases in Africa. Because insufficient serum was available, only part of his patients received it, and some of these only small amounts. Moreover, many of those receiving serum were very ill and some died within a few hours. The mortality was 54 per cent in 139 cases who received serum, whereas 82 per cent of 102 patients who received no serum died. When it is realized that serum in this experience was reserved for those most seriously ill, it is quite obvious that it had considerable effect on the mortality.

The therapy of meningococcus meningitis is being modified rapidly at the present time as a result of the demonstration that sulphanilamide is a very effective agent for combating meningococcal infections. This drug has been used experimentally in treating mice infected with meningococci, both alone and in conjunction with serum, by a number of investigators including Buttle, Gray, and Stephenson (76), Proom (77), and Branham and Rosenthal (78). Good results have been obtained from the use of this drug in the treatment of meningococcus meningitis in man (79, 80, 81, 82, 83). Although it has appeared to be thoroughly effective in man when used alone, it is possible that we may ultimately find a better effect from the combined use of sulphanilamide and anti-serum.

Prophylaxis of Meningitis by Vaccination. If our reasoning is correct that infection of the meninges by the meningococcus is the result of a blood-borne infection, and since the blood of some individuals, at least, possesses meningococcidal power, then it would seem to be possible to protect against the disease by vaccination. Prophylaxis by vaccination should be directed toward the production of antibacterial immunity in the individual. For this purpose, of course, a properly prepared vaccine would be essential, but at the present time our information does not permit us to state categorically how such a vaccine should be made. On the basis of our general knowledge, however, one is justified in sug-

gesting that it should be made from antigenically complete, virulent organisms and should be killed by methods that will not injure its antigenic composition. Silverthorne (84) showed that it is possible to induce bactericidal power in the blood of children by the injection of a vaccine prepared from a freshly isolated virulent strain.

It is very difficult to carry out a convincing epidemiological study to demonstrate the value of vaccination because of the low incidence of meningitis in a given population, even during an epidemic. Moreover, in many of the older attempts in this direction it is quite possible that the most effective form of vaccine was not employed.

A number of attempts have been made to vaccinate large numbers of individuals, but the results, up to the present time, have not been entirely convincing. Zrůnek and Feierabend (85) conducted such a study in the Czechoslovak army. A group of soldiers was vaccinated by three doses of vaccine, consisting of 2000, 4000, and 4000 million organisms prepared from recently isolated strains and killed by heating at 60° C. for one hour. Among 21,280 vaccinated soldiers five developed meningitis and three died, while among 19,684 controls there were six cases of meningitis with three deaths. Riding and Corkill (86) carried out a similar experiment among the natives in the northern Sudan. No significant protection was afforded by their vaccines. Pergher and Portois (87) vaccinated a large number of natives in Africa with a vaccine prepared from local strains. Their results seemed to indicate some benefit from the procedure. Ismaïl (88) reported favorable results following prophylactic vaccination of large numbers of individuals in Turkey. Obviously, more evidence is required before one can form an opinion of the value of prophylactic vaccination in meningitis.

Hemophilus Influenzae Meningitis

We have already indicated that the influenza bacillus is a frequent cause of meningitis in young children. The possibility of developing serum therapy for this disease was in an unsatisfactory state prior to the observations of Pittman (89) and Ward and Wright (90, 91), who showed that meningeal strains of this organism grew in smooth colonies, were encapsulated, and secreted a soluble specific substance. It was likewise shown by these investigators that most meningeal strains formed a single or homogeneous serological group. Fothergill and Chandler (92) found that when a series of serologically homogeneous meningeal strains were dissociated into their avirulent variants, the latter formed a heterogeneous group similar to ordinary respiratory strains. It was found by Chandler, Fothergill, and Dingle (93) that the virulence of ordinary respiratory strains for mice was also similar to that of rough or dissociated meningeal strains.

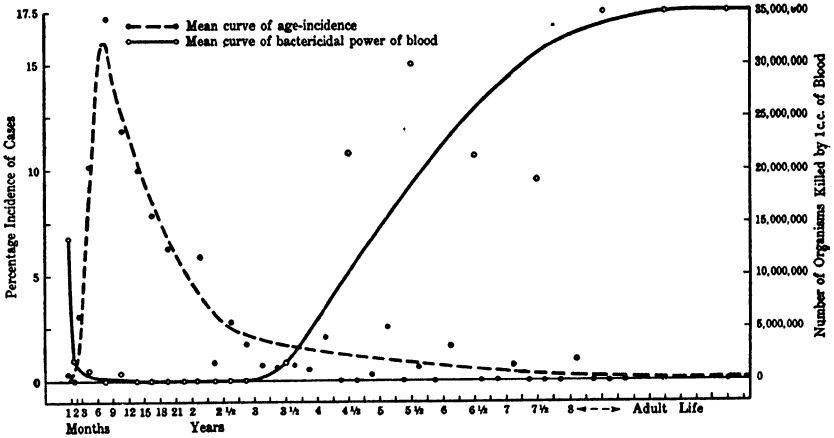
Ward and Wright showed that the immune mechanism resulting in the destruction of influenza bacilli was a complemental bacteriolysis and, according to Fothergill (94), complement is rarely present in the cerebrospinal fluid of patients with this type of meningitis. Ward and Fothergill (95) described the treatment of five patients with a mixture of immune serum and human complement. While none of these patients survived, the spinal fluid was sterilized in some of them for variable periods of time. It appeared that there was a marked tendency for the development of localized lesions which resulted in the ultimate death of the patients. This has been the subsequent experience as these studies continued.

In pursuing these investigations further, Fothergill (2) reported the results of treating 201 cases. Of these, 31 recovered, resulting in a mortality of 84.6 per cent. Silverthorne, Fraser, and Snelling (96) reported 10 recoveries in 35 cases treated with serum supplied by Fothergill. While these results are not impressive, nevertheless, it is a significant reduction in mortality, which is usually 98 to 100 per cent.

The method of serum therapy used by Fothergill is as follows: Serum without complement is given intravenously once daily for two days in doses of 30 cc. for an infant and 30 to 50 cc. for older children. Intravenous serum is given to overcome the bacteriemia that is present in practically all cases. A mixture of antiserum and complement is given intrathecally twice daily for as long as is indicated. This mixture ordinarily consists of two parts antiserum and one part complement. The total amount of mixture given is dependent upon the amount of cerebrospinal fluid withdrawn. Fresh adult human serum is used as complement and is mixed with the antiserum at the time of each treatment. Dingle, Fothergill, and Chandler (97) have shown that the species of animal from which complement is obtained is of considerable importance. They found that guinea pig complement was unable to activate anti-influenza antibody produced in horses. Human complement, however, was effective in activating this antibody.

An explanation for the age distribution of influenza bacillus meningitis was given by Fothergill and Wright (16). They showed that the defibrinated blood of adults usually possessed considerable bactericidal power for meningeal strains. The blood of children between two months and three years of age, the age-period during which the majority of cases occur, seldom exhibited this property. As age advanced, the blood of an increasing percentage of individuals showed bactericidal activity. Umbilical cord blood and the blood of newly born infants possessed considerable bactericidal activity which was probably due to passively transferred antibody from the mother. This property disappeared from infants' blood during the first six weeks of life. The bactericidal power

of the blood of newly born infants is associated with an absence of the disease during the first two months of life. The following figure represents these relationships:



THE RELATION OF AGE INCIDENCE OF H. INFLUENZAE MENINGITIS TO THE BACTERICIDAL POWER OF HUMAN BLOOD AT DIFFERENT AGES AGAINST A SMOOTH MENINGEAL STRAIN OF H. INFLUENZAE.

Taken from Fothergill and Wright (16).

Pneumococcus Meningitis

It is only logical that numerous attempts should have been made to treat patients with this disease with antipneumococcal serum. While occasional cases of recovery have been reported, the results in general have been extremely disappointing. Finland, Brown, and Rauh (98) have reported recoveries in six cases treated by the administration of immune serum and complement, together with large doses of sulphanilamide.

Shaffer, Enders, and Wilson (99) reported on the treatment of a group of patients with Type III pneumococcus meningitis in which a potent Type III rabbit serum was injected intrathecally in patients whose body temperature was raised artificially by the application of radiant heat to 105° F. or higher for periods of several hours. This procedure was the result of the observation of Enders and Shaffer (100) that certain strains of this organism were unable to grow at a temperature of 41° C. They were able to correlate the virulence of strains for rabbits with their capacity to grow at this elevated temperature. While none of the patients which they treated survived, nevertheless, certain evidences were obtained indicating a favorable effect, that is, soluble type-specific antigen disappeared rapidly from the cerebrospinal fluid and a marked reduction in the numbers of organisms occurred.

Hemolytic Streptococcal Meningitis

We have no effective form of serum therapy for this uniformly fatal disease. However, sulphanilamide has been shown to be an effective therapeutic agent. A number of cases have been reported (101, 102, 103, 104) as cured since the advent of this drug. The methods of administering the drug have been described in our chapter on hemolytic streptococcal infections.

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CHAPTER XXV

PNEUMONIA

PNEUMONIA is an acute inflammatory process involving one or more lobes of the lungs, characterized, in the majority of cases, by an explosive onset and an abrupt termination after a stormy course of from seven to ten days. Not only is the disease quite common but the mortality rate is high. It is generally estimated that pneumonia is about the seventh most common cause of death. Being typically a respiratory disease, it has a seasonal peak of distribution corresponding to that of other acute upper respiratory tract infections, *i.e.*, its most frequent occurrence is during the winter and early spring months.

In the past emphasis was laid upon the anatomic character of pneumonia. Two main classes are recognized, lobar and bronchopneumonia. Lobar pneumonia is the classical type with a sudden onset and typical termination. It is the type most commonly occurring during the "middle" period of life. Bronchopneumonia is, anatomically, of a more patchy and scattered distribution, with more widespread interstitial involvement than in the true lobar variety. Bronchopneumonia occurs most commonly during the extremes of life, occurring with great frequency in infants under two years of age and in the aged. It is a frequent secondary terminal event in many conditions, particularly chronic debilitating diseases. It often occurs during the course of other acute infectious diseases such as influenza, measles, and whooping cough.

At the present time less emphasis is placed on the anatomic classification of pneumonia. Since both lobar and bronchopneumonia are susceptible to treatment with specific serum, and since sera are now available for a number of the higher types, the only rational classification for clinical management is an etiological one. Therefore, every effort should be devoted to learning as soon as possible the nature of the inciting agent in each individual case regardless of the anatomical character of the disease.

Practically all cases of true lobar pneumonia are caused by the pneumococcus, although the true clinical picture may rarely be caused by other organisms. The following figures, taken from the Rockefeller Monograph (1), describe the frequency of the more common infecting

organisms in 480 cases in which this was determined. In this series the pneumococcus was responsible for about 96 per cent of the cases.

Diplococcus pneumoniae	454 cases
Friedländer's bacillus	3 cases
Hemophilus influenzae	6 cases
Streptococcus pyogenes	7 cases
Streptococcus mucosus	1 case
Staphylococcus aureus	3 cases
Mixed infections	6 cases
Total	480 cases

In bronchopneumonia the situation is somewhat different. In this disease the distribution of the inciting organisms follows a much less standard pattern. In general, the organism causing the pneumonic process is the same as that in the upper respiratory passages. Thus, the hemolytic streptococcus and the Type III Pneumococcus, as well as members of the higher types of pneumococci, are the common causes of bronchopneumonia. Types I and II, which together account for about 50 per cent of lobar pneumonia, are infrequent causes of the lobular type. This does not, of course, invalidate the necessity and urgency for determining the type of infecting organism in every case of bronchopneumonia, particularly since sera are now available for a number of the higher types. A not insignificant number of cases of bronchopneumonia may be benefited by serum treatment.

Types of Pneumococci. One of the most important advances in the development of our knowledge of the management and epidemiology of pneumonia has been the classification of these organisms into their numerous individual serologic races. As early as 1897, Bezançon and Griffon (2, 3) clearly recognized that the bacterial species, pneumococcus, was composed of a number of distinct varieties as determined by serological reactions. During the next few years numerous investigators confirmed these observations concerning the serological diversity of pneumococci. A distinct contribution was that of Collins (4), in 1905, who showed that *Pneumococcus mucosus* was a distinct variety and that this organism was definitely related to the pneumococci rather than to the streptococci.

Neufeld and Haendel (5), in 1910, demonstrated the significance of the serological grouping of pneumococci. They described a "Pneumococcus I" or "typical" pneumococcus and another type or "atypical" pneumococcus which corresponded to our present Type II. They showed that potent serum for one type did not protect mice against becoming infected by another type. It was pointed out that to be effective in therapy a potent, type-specific serum would have to be used, a principle which still holds good today.

Finally, in 1913, Dochez and Gillespie (6) divided the pneumococci

into four groups. Their Types I and II, which accounted for over 60 per cent of the strains tested, corresponded to the two types described by Neufeld and Haendel. Type III was made up of the *Pneumococcus mucosus* variety, and all other organisms which could not be placed into one of these three fixed types were designated as Group IV. This remained the standard system of classification until the past few years, when Group IV was broken up into its separate serological types.

From time to time efforts were made to separate Group IV into its separate serological types. Studies in this direction were made by Olmstead (7), Cooper, Mishulow, and Blane (8), Griffith (9), and others. In addition, a number of investigators, including Avery (10) and Stillman (11), had described a number of sub-types of Type II. Furthermore, various writers had, throughout this time, described various individual strains having certain distinctive properties. Sugg (12) and his associates described an organism related to Type III, although it had distinct immunological properties of its own.

Definite order was finally given to the classification of pneumococci by the painstaking studies of Cooper (13, 14) and her associates. They described 29 distinct types of pneumococci in addition to the three fixed types and showed that a specifically reacting monovalent serum could be prepared against each of them. They further correlated their types with the previously described odd types. For example, the atypical Type III described by Sugg *et al.* corresponded to Cooper's Type VIII. Cooper and her associates designated these new types by the Roman numerals IV through XXXII.

The Cooper system is the generally accepted method of pneumococcal classification at the present time. Having a system whereby the heterogeneous organisms, other than the three fixed types, can be classified is of great value. In addition to the valuable epidemiological studies which have been possible, it permits the extension of type-specific serum therapy to a large number of patients who would otherwise be denied it. Serum is now available for infections due to the more common of the higher types.

Cooper and her co-workers found that practically all strains of pneumococci isolated in New York City could be classified into one or another of these 32 types. It is only natural, however, that strains should be occasionally isolated, particularly from other localities which were not identified within this grouping. It is the general experience, nevertheless, that the vast majority of strains can be typed by this system. From time to time other well-defined types will undoubtedly be added.

The Distribution of Pneumococcal Types in Pneumonia. Heffron (15) has collected figures on the distribution of the three fixed types of pneumococci in various parts of the world.

Types I and II are responsible for a little over 50 per cent of all cases of lobar pneumonia, and the types comprising Group IV account for about 35 per cent of the cases. This is important since it emphasizes that one out of every two patients, under ideal management, has a greatly increased chance of recovery as a result of serum therapy. This opportunity is gradually being extended to a still greater proportion of cases as sera for certain types of Group IV are being developed.

It must be emphasized, of course, that these figures represent averages from large numbers of cases. It is well recognized that the percentage distribution of the different types varies from season to season and from locality to locality. In addition, isolated epidemic outbreaks due to a single type have been described.

TABLE I

OCCURRENCE OF PNEUMOCOCCUS TYPES IN DISEASE

TYPE	A LOBAR PNEUMONIA IN ADULTS *		B ATYPICAL PNEUMONIA IN ADULTS		C EMPYEMA ON ADMISSION IN ADULTS		D PNEUMONIA AND/OR EMPYEMA IN CHILDREN		E FOCAL INFECTIONS (NO PNEUMONIA)		F CARRIERS AND RESPIRATORY INFECTIONS WITHOUT PNEUMONIA		ALL CASES	
	No.	Per Cent	No.	Per Cent	No.	Per Cent	No.	Per Cent	No.	Per Cent	No.	Per Cent	No.	Per Cent
I	654	29.3	28	4.5	27	39	118	55.1	34	14.0	12	3.9	873	23.7
II	294	13.2	15	2.4	10	15	9	4.2	17	7.0	8	2.6	353	9.6
III	335	15.0	99	16.0	5	7	6	2.8	76	31.3	51	16.6	572	15.5
IV	46	2.1	18	2.9	1	1	4	1.9	6	2.5	10	3.2	85	2.3
V	191	8.6	30	4.8	5	7	19	8.9	17	7.0	6	1.9	268	7.3
VI	28	1.3	30	4.8	3	4	8	3.7	10	4.1	18	5.8	97	2.6
VII	128	5.7	32	5.2	4	6	2	0.9	10	4.1	19	6.2	195	5.3
VIII	159	7.2	68	11.0	8	12	4	1.9	22	9.1	18	5.8	279	7.6
IX	30	1.3	12	1.9	2	3	3	1.4	2	0.8	7	2.3	56	1.5
X	37	1.7	40	6.5	1	1	2	0.9	5	2.1	17	5.5	102	2.8
XI	29	1.3	18	2.9	0	0	4	1.9	8	3.3	8	2.6	67	1.8
XII	38	1.7	8	1.3	1	1	4	1.9	3	1.2	3	1.0	57	1.5
XIII	13	0.6	12	1.9	0	0	0	0	2	0.8	6	1.9	33	0.9
XIV	51	2.3	24	3.9	1	1	15	7.0	7	2.9	7	2.3	105	2.9
XV	7	0.3	6	1.0	0	0	0	0	0	0	5	1.6	18	0.5
XVI	4	0.2	8	1.3	0	0	1	0.5	0	0	1	0.3	14	0.4
XVII	19	0.9	20	3.2	0	0	2	0.9	1	0.4	7	2.3	49	1.3
XVIII	38	1.7	32	5.2	0	0	1	0.5	4	1.6	20	6.5	95	2.6
XIX	19	0.9	12	1.9	0	0	4	1.9	5	2.1	7	2.3	47	1.3
XX	36	1.6	35	5.7	0	0	4	1.9	3	1.2	15	4.9	93	2.5
XXI	9	0.4	9	1.5	0	0	1	0.5	3	1.2	4	1.3	26	0.7
XXII	10	0.4	10	1.6	0	0	0	0	2	0.8	4	1.3	26	0.7
XXIII	2	0.1	3	0.5	0	0	1	0.5	0	0	4	1.3	10	0.3
XXIV	0	0	3	0.5	0	0	0	0	0	0	5	1.6	8	0.2
XXV	3	0.1	2	0.3	0	0	0	0	1	0.4	1	0.3	7	0.2
XXVII	1	0	0	0	0	0	0	0	0	0	1	0.3	2	0.1
XXVIII	0	0	4	0.6	0	0	0	0	1	0.4	1	0.3	6	0.2
XXIX	4	0.2	4	0.6	0	0	0	0	2	0.8	3	1.0	13	0.4
XXXI	3	0.1	0	0	0	0	0	0	0	0	2	0.6	5	0.1
XXXII	3	0.1	1	0.2	0	0	0	0	0	0	1	0.3	5	0.1
Neg. 1–XX	35	1.6	35	5.7	1	1	1	0.5	2	0.8	37	12.0	111	3.0
Neg. 1–XXXII	3	0.1	1	0.2	0	0	1	0.5	0	0	0	0	5	0.1
Totals	2229	100	619	100	69	100	214	100	243	100	308	100	3682	100

* Includes cases with lobar pneumonia in one or more lobes and atypical in other parts.

Taken from Finland (17).

With regard to the higher types, we do not as yet have such extensive data concerning their percentage distribution and the mortality due to the individual types. Sufficient data are at hand, however, to indicate that infections with certain types occur more frequently than with others. The more common of these in causing lobar pneumonia in adults in the order of their frequency appear to be VIII, VII, and V, as judged from collected figures. These three types account for roughly 20 per cent of cases of lobar pneumonia.

The distribution of pneumococcal types in bronchopneumonia is quite different from that described above. In this case the distribution of types corresponds closely to their distribution in the upper respiratory tract of carriers. Infections with Types I and II are not common. Considering all the types, the distribution in order of frequency is as follows in collected series of cases: III, VIII, XVIII, X, V, and VII. The above order of frequency of types of pneumococci in lobar and bronchopneumonia was taken from the monograph of White (16) and his collaborators.

The table on the opposite page taken from Finland (17) describes the distribution of pneumococcal types in a group of 3682 patients observed at the Boston City Hospital. Approximately a third of these cases occurred prior to 1932, at which time typing sera were available for Types I through XX only. The author has omitted Types XXVI and XXX because of their probable identity with Types VI and XV respectively.

The Distribution of Pneumococcal Types in Children. There are certain differences in the etiology of pneumonia in infants and children as compared to adults. Data concerning large series of cases have been published by Nemir, Andrews, and Vinograd (18), Bullowa and Greenbaum (19), and Andrews (20). From these data certain facts are apparent. Other organisms, particularly the hemolytic streptococcus, are common causes of pneumonia in the younger age group. It would appear that the pneumococcus is the etiologic agent in about 65 per cent of the cases and all other organisms in about 35 per cent. Among cases due to the pneumococcus, the distribution of types is quite different from that in adults. Taking these series together, the different types occurred in the following order of frequency: XIV, I, VI, XIX, V, IV, III, and VII.

There is a change in the distribution of types depending on the age of children, as shown in the following table taken from Nemir, Andrews, and Vinograd (18). Type I is not common in children under two years of age. As age advances, this type rapidly becomes more common so that in later childhood it is the most common type, corresponding in the frequency of its distribution to that in adults. Type XIV is by far the most common type in infants. Its frequency decreases as age advances.

TABLE II

PREDOMINANT PNEUMOCOCCI IN PATIENTS WITH LOBAR PNEUMONIA

TYPES	ALL AGES UNDER 13 YEARS		UNDER 2 YEARS		2 TO 13 YEARS	
	Patients	Percentage	Patients	Percentage	Patients	Percentage
I	135	17.8	22	7.7	113	23.9
VI	71	9.3	32 *	11.2	39	8.2
XIV	149	19.6	103	36.0	46	9.7
V	44	5.9	7	2.4	37	7.8
VII	32	4.4	12	4.2	20	4.2
XIX	22	2.9	15	5.0	7	1.5
Totals	453	59.9	191	66.5	262	55.3
Other organisms than pneumococci	75	10.0	19	6.6	56	11.9
Number of cases †	758	100.0	286	100.0	472	100.0

* Mixed with hemolytic streptococci or with other pneumococci.

† Data for 230 patients not charted; these had lobar pneumonia associated with other types of pneumococci than those listed.

Taken from Nemir, Andrews, and Vinograd (18).

The Typing of Pneumococci. The first essential to the specific treatment of patients with lobar pneumonia is a rapid and accurate typing. The most common source of material for this purpose is sputum, and in its collection certain precautions should be taken to minimize the chances of error. It is essential to have the patient rinse out his mouth several times with sterile water or physiologic salt solution to reduce the number of contaminating organisms. The patient should then be encouraged to cough in order to raise sputum from the depths of the respiratory tract. This step is extremely important, and failure to accomplish it is one of the difficulties in doing accurate typings on the sputum of infants and young children. The specimen of sputum is collected in a clean glass bottle or sputum cup and taken directly to the laboratory.

The purulent material may be washed several times with sterile saline before typing, depending on the typing technique to be employed. It is always important to prepare a Gram-stained smear of the material to ascertain if organisms are numerous or very few. If organisms are very scarce, certain of the typing procedures may not be successful. The more commonly employed methods of typing are as follows:

Neufeld "quellung" method. In 1902, Neufeld (21) noted that when pneumococci were mixed with homologous immune serum, a pronounced swelling of the capsule occurred. This phenomenon was not applied to practical typing until 1931, when Neufeld and Etinger-Tulczynska (22) employed it to identify the types of pneumococci in mixed cultures.

This principle was soon applied to the rapid typing of pneumococci in sputum by Armstrong (23, 24) and Logan and Smeall (25). The reliability of the method was thoroughly demonstrated by the comparative studies of Sabin (26), Beckler and MacLeod (27), and Cooper and Walter (28).

The Neufeld technique as applied to all 32 types of pneumococci by Beckler and MacLeod (27) of the Massachusetts Department of Public Health is as follows:

"Upon receipt of the sputum at the laboratory, stained liquid mounts of the specimen are mixed with rabbit antisera (Types I to XXXII) used undiluted. Combinations of monovalent antisera (rabbit) are used instead of making thirty-two preparations of the sputum with the thirty-two monovalent sera. The combinations of sera used are the following:

Type I
A, Types II, IV, V, and VII
B, Types III and VIII
C, Types IX, XI, XIII, and XV
D, Types VI*a*, VI*b*, XVII, and XVIII
E, Types XII, XIV, XVI, and XXVIII
F, Types X, XIX, XX, and XXI
G, Types XXII, XXIII, XXIV, and XXV
H, Types XXVII, XXIX, XXX, XXXI, and XXXII

"Nine loopfuls of sputum are placed approximately one inch apart on a 9 × 2 inch plane glass slide; to each drop is added two loopfuls of the antiserum, *i.e.*, the first drop is mixed with Type I antiserum, the second drop is mixed with combined serum A (II, IV, V, and VII), the third drop with B, etc. These preparations are stained with Loeffler's alkaline methylene blue (two drops to each mixture) and are covered at once with cover slips to prevent drying. Examination is made with the oil immersion lens, with the light dimmed. When a positive reaction occurs, which is usually within a few minutes, there is a decided swelling of the capsule of the pneumococcus present. The swollen capsule is of a light greenish-gray color, is much less translucent than one that is not swollen, and has a very definite outline which is one of the most characteristic features of a positive reaction. In the preparations in which no reaction is evident, the capsule of the pneumococcus appears as a halo of refracted light. In all preparations the body of the pneumococcus stains a definite blue. If the reaction is observed in drop I, then the pneumococcus present is Type I and can be reported immediately; if the reaction is observed in drop 3, for example, the test is repeated using two drops of the sputum mixed with Type III and Type VIII undiluted monovalent rabbit sera, respectively; if the reaction is observed in drop 9, five loopfuls of the sputum are mixed with Types XXVII, XXIX, XXX, XXXI, and XXXII undiluted monovalent sera, respectively. Should no reaction be seen on the first examination, the preparations are re-examined at the end of thirty minutes.

"When dealing with sputa containing many Type III organisms, it has been necessary, occasionally, to dilute the sputum with saline before any "quellung" of the pneumococcus capsule became evident. In such instances,

when we have used the regular Neufeld technique, the organisms formed large masses which were surrounded by much precipitated material; and no definite swelling of the capsules could be seen. However, upon dilution of the sputum with saline and the test repeated, the individual diplococci have shown a typical positive reaction.

"The age of the sputum specimen apparently makes little difference in the successful application of this technique. Positive results can be demonstrated on specimens 48 hours or more after collection."

This typing technique has been an important advance in the better management of patients with pneumonia. It is no longer necessary for the patient to wait 6 to 24 hours for the result of typing by one of the older methods before receiving serum treatment.

Mouse inoculation and typing. For many years the isolation of pneumococci by the inoculation of sputum into the peritoneal cavity of mice with the subsequent typing of the organisms in the peritoneal exudate was a standard and widely employed procedure. Although the results obtained by this method were accurate, it had the disadvantage of being time-consuming. The recently developed more rapid methods have gradually supplanted it. However, it still serves a useful purpose under certain circumstances. When organisms are too few in a specimen of sputum to permit the use of the Neufeld method, the mouse method will often yield a positive result. It is a useful and standard procedure for checking other methods.

The details of the method can be found in any standard laboratory manual or textbook of bacteriology.

Slide agglutination typing method. Sabin (***29***, ***30***). "One cc. of a fresh sample of sputum is injected intraperitoneally into a mouse. Three to four hours after injection some of the peritoneal fluid is obtained by capillary puncture. A glass slide is marked off into four parts, and a minute drop of the peritoneal fluid is expelled onto each one of the four partitions. The first is smeared with saline for control, and the others with a loopful of a 1–10 dilution of Type I, of Type II, and of Type III, diagnostic serums respectively. This dilution of serum is chosen to largely eliminate group agglutinins. The smears are made thin, so that they dry rapidly; they are then stained for one-half minute with a fuchsin solution (10 cc. saturated alcoholic solution of basic fuchsin plus 90 cc. distilled water). The stain is washed off in running water, and the smears are examined with the oil-immersion lens. If a specific agglutination reaction is observed in one of the smears with diagnostic serum, the organism is of the corresponding type. If no reaction occurs in any of the smears, and pneumococci are clearly seen, a diagnosis of Group IV is made. In performing a complete typing for all the 32 types, the sera may be combined into 8 groups; for example, Group A may contain the sera of Types I–IV in final dilution of 1–10 or 1–20; Group B, sera Types V–VIII, etc. When agglutination occurs in one of the groups, four smears are made with the constituent sera of that group, to determine the one type; with no clumping in any of the groups, the pneumococcus is termed unclassified.

" Naturally occurring clumps of organisms differ in appearance from those produced by specific agglutination; they can be recognized by their occurrence in the saline control smear as well. Unless a fresh sample of sputum is used many of the organisms will have undergone autolysis and therefore more time must be allowed for growth. Since the mouse is not killed, another typing can be done if the first one should show insufficient organisms, and after death of the mouse the type may be confirmed. In the case of Type III sufficient organisms for a microscopic typing are present as early as two hours after injection. The appearance of the specific reaction with Type III differs from that obtained with other types of pneumococci, primarily on account of the larger size of the capsule."

Other typing procedures that are occasionally employed are the urine precipitation test of Dochez and Avery (31) and the precipitation test of Krumwiede and Valentine (32) on the clear filtrate obtained from coagulated sputum. These tests are dependent upon the fact that the soluble specific substance is present in these secretions.

Application of Typing Methods to Other Materials. It is often necessary to type pneumococci that are present in various body fluids, and for this purpose the methods described above are suitable. The Neufeld method can often be applied directly to empyema fluid, providing the organisms are present in sufficient numbers and that they have not undergone much autolysis. If direct typing cannot be done, 0.5 cc. of the fluid may be injected intraperitoneally into a mouse and the typing carried out subsequently in the usual manner. In addition, empyema fluids should always be cultured on suitable media for final identification of the organism.

In pneumococcal meningitis it is usually possible to type the organism directly from the cerebrospinal fluid because in the majority of patients they are present in this secretion in enormous numbers. The Neufeld technique can be applied directly to the fluid and a typing result can often be obtained within half an hour of lumbar puncture. The Sabin slide agglutination may be similarly applied directly to the cerebrospinal fluid. If organisms are very scarce, it is necessary to culture or inject the fluid into a mouse before typing can be done.

Typing can nearly always be done directly on positive blood cultures. Some of the supernatant broth containing organisms in suspension can be aspirated from the flask, centrifuged, and the organisms resuspended in physiological salt solution. This suspension can then be tested by either the Sabin slide agglutination or by macroscopic agglutination. If such cultures are examined while very young, it is often possible to do a satisfactory Neufeld typing. It should be remembered, however, that pneumococci may lose their capsular material fairly rapidly in broth cultures so that after 24 to 36 hours' incubation it may be impossible to get a satisfactory result by the Neufeld method.

It is important to emphasize that pneumococci in positive blood cultures should always be typed. In rare instances this may be the only source of organisms for typing (particularly in infants and young children where a satisfactory sputum typing is difficult). In all cases blood culture typing provides a valuable check on the results of sputum typing. A discrepancy is occasionally revealed which is of the utmost importance to the patient's welfare.

The Treatment of Patients. Sensitivity Tests. Administration of Serum and Dosage. The phenomena concerned in serum disease, sensitivity tests, etc., have been discussed elsewhere in this text; however, certain practical problems may be reiterated here, since in the specific treatment of pneumonia, antiserum is always administered by the intravenous route — the most dangerous route in so far as untoward reactions are concerned. It is extremely important to select patients carefully from the point of view of the possibility of reactions.

In general there are several classes of patients who must be dealt with. The most likely of these to develop severe or fatal anaphylactic shock are patients with a history of asthma or vasomotor rhinitis definitely due to horse emanations. Such patients should not be treated with serum. Patients of this character have been treated, but severe asthma and anaphylactic shock have occurred. The danger of sudden fatal shock is always imminent.

Serum can generally be administered to patients with a history of asthma or hay fever not related to horse emanations if the sensitivity tests are negative and if it is given with the utmost caution. Patients with other evidence of allergy, such as a family or personal history of eczema, urticaria not due to serum disease, angioneurotic edema, etc., probably do not represent an increased hazard providing their sensitivity tests are negative. Serum should not be administered in this group unless both skin and eye tests are negative.

The third group of patients are those with a history of previous administration of horse serum in some form such as diphtherial toxin-antitoxin mixture, tetanus, Dick or diphtheria antitoxin, etc., but without a history of other evidence of allergic manifestation. Following a first injection of foreign serum a certain proportion of patients develop serum disease in from five to ten days. The frequency of serum disease and its severity appear to be related to the dosage of serum. Sensitivity is of maximum intensity during the first few months after serum administration. If a second dose of serum is given within six months to two years, it is generally followed by the development of an accelerated serum disease. When many years have elapsed, the majority of patients will have lost their sensitivity, and serum disease will occur after the usual incubation period. Sensitiveness from serum administered a number of years previously is

much less dangerous than spontaneous sensitiveness such as in asthma or hay fever.

Patients with asthma or hay fever definitely due to horse emanations need not be tested for sensitivity, since serum therapy is already contraindicated by the history. Indeed, performing an intradermal sensitivity test in such individuals may be followed by a sudden fatal shock.

In all other patients it is preferable to do both the eye and skin test. The eye test is much less sensitive than the skin test and when positive is a definite contraindication for intravenous serum therapy. A strongly positive skin test consisting of a large wheal with pseudopods in an area of erythema is likewise a contraindication for intravenous serum. A negative eye test and a weakly positive skin test should be regarded as requiring the utmost caution in serum administration. The first intravenous dose should be extremely small and should be preceded by a subcutaneous injection of 5 to 15 minims of adrenalin chloride (1 : 1000 dilution). The eye test is not a satisfactory procedure in small children. The serum is apt to be washed from the conjunctival sac by crying.

In all procedures involving the injection of serum into a patient the physician should have adrenalin at hand. It should not be in the bottle. It should be loaded in a syringe with a needle to be ready for immediate use.

In addition to patients who are a serious risk because of properly demonstrated sensitivity, those *in extremis* should not be given serum. There is little likelihood that it would help them and even a very mild chill or allergic reaction might tip the balance against them.

Those patients who have a severe shock-like reaction, an asthmatic attack, or a severe thermal reaction should not be given additional serum. These reactions are a contraindication to further serum therapy.

Chills and thermal reactions which are probably unrelated to hypersensitive phenomena occur in about 20 per cent of patients following the intravenous injection of antipneumococcic serum. Concentrated and refined sera produce fewer chill reactions than raw sera. Certain lots of serum may produce more chills than others. The chill-producing factor in antipneumococcic serum is unknown, and attempts to remove it have not been entirely successful. Barnes and Kramer (33) described a test for chill-producing lots of serum which consists of the intravenous injection of the serum in monkeys and observing the animals for chills and a rise in temperature. Barnes and Robinson (34) further modified this test and found that by its application the incidence of chills in patients has been reduced by about 50 per cent.

In the actual treatment of patients with pneumonia the antibody solution is always given intravenously. While it is desirable to administer

the total therapeutic dose as quickly as possible, it is imperative to proceed cautiously and give it in divided injections.

The more usual plan is to give a first or trial injection of 2 cc. This is given very slowly, at a rate not to exceed one cubic centimeter per minute by the clock. Since bacteriemia is such an important guide for the amount of therapy, it is not only convenient but imperative to take a blood culture at the time of this first injection of serum. This may be done as follows: a venipuncture is done with an appropriate needle attached to a small syringe. Five to 10 cc. of blood are withdrawn and the syringe is disconnected from the needle, leaving the needle in place in the vein. The blood is then inoculated into appropriate media which should be at hand. Another small syringe, loaded with serum warmed to body temperature, is connected to the needle in the vein and the serum injected slowly. It is advisable to make certain that the needle is still in place before injecting the serum by aspirating a small amount of blood. Blood for culture should never be taken by aspirating blood back into the syringe used in giving serum.

Should a reaction develop during the course of this or any subsequent injection, the procedure should be interrupted instantly and proper emergency treatment instituted. Otherwise the patient is carefully observed for the development of reactions during the next two hours. If none occur, the second injection of serum is given, which usually consists of the remainder of the serum in the vial from which the first 2 cc. were taken (generally about 8 to 13 cc.).

After an additional two hours the third injection, usually consisting of two vials or 30 cc., is given. By this time an average therapeutic dose of 60,000 to 100,000 units will have been administered. Actually, of course, the volume of serum injected will depend on its potency. The physician should always note this with each vial of serum, since dosage is always given in terms of units. With a weak serum a larger volume is necessary. Most sera at the present time have a potency of about 2000 units per cubic centimeter. Additional injections of serum will depend upon certain indications which will be discussed presently.

The average dose of serum in Type I cases, according to Lord and Heffron (35), is about 60,000 to 100,000 units. For Type II cases the usual dose is 100,000 units. At the present time we have insufficient statistical data concerning the treatment of patients infected with the higher types of pneumococci to make definite statements of dosage. In general it may be said that doses of about 100,000 units should be given to uncomplicated cases. This problem will be dealt with in more detail when we consider the results of serum therapy.

There are a number of factors which influence the mortality from pneumonia and the outcome of serum therapy. In many instances the

dosage of serum may be increased to compensate, in part at least, for these adverse circumstances. One of the most important of these factors is the presence of bacteriemia. Lord and Heffron gave figures on a large group of patients who received no specific treatment. In 329 Type I cases with negative blood cultures the mortality was 18.5 per cent, whereas in 213 Type I cases with positive blood cultures the mortality was 70.9 per cent. In a similar series of Type II cases the mortality in the group with negative blood cultures was 13.3 per cent, while in those with positive cultures it was 76.1 per cent. There is some evidence of a relationship between the mortality and the amount of bacteriemia. It is important, therefore, to make pour plates when doing blood cultures in order to estimate the number of organisms per cubic centimeter of blood.

It has long been recognized that among the three fixed types of pneumococci there was a variation in mortality depending on the type in untreated cases. On the average the mortality in Type I infections is 30 per cent, in Type II 43 per cent and in Type III 50 per cent. Among the higher types where collected figures are reasonably large there appears to be a variation in mortality from about 25 to 35 per cent, with Type XIV accounting for the highest mortality rate.

All are agreed that a most important factor in outcome is the earliness in the course of the disease that serum is administered. Cecil (36) collected a series of 160 cases of Type I pneumonia treated within the first 24 hours of onset. The fatality rate was 5 per cent, whereas an average fatality rate for Type I cases treated within the first four days of onset is about 12 per cent. The following table taken from the report (37) of the Massachusetts study also illustrates the importance of early treatment.

TABLE III

Day of Illness Treatment Begun	Cases	Died	Per Cent Fatality Rate
1	50	3	6.0
2	162	16	9.9
3	165	13	7.9
4	127	24	18.9
Total	504	56	11.1

Taken from Heffron and Robinson (37).

While the importance of early treatment cannot be overemphasized, it is evident that no time limit can be set beyond which it can be definitely stated that serum will do no good. In a series, collected from the literature by Lord, of 711 Type I cases that were treated after the fourth day, Lord and Heffron reported a mortality of 19.7 per cent. The

Massachusetts Department of Health, in their continuing pneumonia study, have abolished the regulation restricting the use of serum to the first four days of illness.

There are a number of additional factors which influence the mortality from pneumonia. Fatality rates are much higher in persons over fifty years of age than in younger individuals. Pneumonia is an especially serious disease in persons with various chronic disturbances such as cardiovascular-renal disease, in chronic alcoholics, and as a complication of operative procedures. It is a serious complication of pregnancy and the puerperium. It is, of course, only natural that the mortality from pneumonia should increase in direct proportion to the amount of pulmonary involvement.

Because of these various factors influencing mortality, it is generally advised that the dose of serum should at least be doubled under the following circumstances:

a. When treatment is begun after the third day.
b. When bacteriemia is known to be present.
c. If more than one lobe is involved.
d. If the patient is over 45 years of age.
e. If the patient is pregnant or in the early part of the puerperium.

Additional serum should be given to patients who continue to have positive blood cultures and to those who fail to show the expected clinical response following the first dose of serum. It is very important to be certain of the accuracy of the typing in patients in this category. The typing should always be checked by a second typing. It is extremely important to type the organism obtained by blood culture. This is an invaluable check under these circumstances.

It should be pointed out that opinions still vary somewhat as to the ideal dose of antipneumococcic serum. This is particularly true with regard to pneumonia due to the higher types of pneumococci, since we do not as yet have sufficient data to justify our being explicit in recommending a particular dosage. Our own recommendations are those of Lord and Heffron which are being followed in the Continuing Pneumonia Program in the State of Massachusetts. A larger dosage is recommended in the report of the Special Subcommittee of the Committee on Public Health Relations of the New York Academy of Medicine (38). This committee advises a dosage of 100,000 units in Type I infections and 160,000 units in Type II infections.

Efforts have been made from time to time to control the dosage of antipneumococcic serum by various immunologic reactions. The patient's serum can be used in an agglutination reaction with the type of organism causing his infection. While this test is simple and quick to perform, it is not particularly reliable under these circumstances. When

it is positive, the reaction usually occurs in low dilution of the serum only. An absence of agglutination does not necessarily indicate insufficient therapy.

Tillett and Francis (39, 40) described a skin reaction with pneumococcal soluble specific substance which indicates the presence of antibody (immunity). Francis (41) employed this reaction as a guide to serum therapy and as a prognostic aid. The test consists of the intradermal injection of 0.1 cc. of a saline dilution containing 0.01 mg. of SSS. The site of injection is observed for 20 to 30 minutes. A positive test, indicating immunity, consists in the development of an area of erythema surrounding a wheal with pseudopodia.

Such procedures as the mouse protection test and the determining of the pneumococcidal power of the patient's blood are not suitable in the control of serum dosage because they are somewhat involved and considerable time is required for their performance.

A demonstrable amount of circulating antibody does not necessarily mean adequate resistance to overcome the local pulmonary infection. This is particularly true in patients who are treated late in the course of the disease. Kline and Winternitz (42) showed that there was considerable impairment of the circulation in the pneumonic lung and as a result there is a barrier to be negotiated by antibody before reaching the actual site of infection. Of interest in this connection also are the observations of Nye and Harris (43), who demonstrated an enormous amount of SSS in consolidated pneumonic lungs. The older consolidated lobes contained the larger amounts. Ward (44, 45) and others have shown that SSS has a specific inhibitory effect on phagocytosis.

Results of Serum Treatment of Pneumonia. There is abundant well-controlled data in the literature to show the striking benefit of the serum treatment of Type I pneumococcus pneumonia (35, 36, 37, 46, 47, 48, 49, 50, 51). It would appear that such therapy reduces the average mortality rate of about 30 per cent to an average mortality rate of about 12 per cent. Failure to use antiserum in this type of pneumonia is to deny the patient the benefit of a very valuable therapeutic agent.

The demonstration that patients with Type II pneumococcic pneumonia may be benefited by serum therapy has not been quite so striking as in the case of Type I infections. Occasional reports have appeared that are discouraging; however, it is now generally agreed that patients with this type of infection may be benefited by serum therapy. As we have already stated, the mortality from this type is about 40 per cent. Serum therapy has reduced this to about 25 per cent.

As we have indicated elsewhere, sera have been developed for use in infections with the more common of the higher types of pneumococci, principally Types V, VII, VIII, and XIV. A number of reports

have appeared concerning the use of these sera in controlled series of cases.

The mortality from Type V infections seems to have been significantly lowered by serum therapy. From reports published by Finland and Tilghman (52), Bullowa (53), Rosenblüth and Block (54), and Benjamin, Blankenhorn, Ruegsegger, and Senior (55), it would appear that the mortality from this type has been reduced by about one half, *i.e.*, from about 32 per cent to 16 per cent.

The mortality in Type VII pneumonia in adults appears to have been reduced by about one half in controlled series of cases published by Kohl and Reitzel (56), Finland *et al.* (57), Benjamin *et al.* (55), and Bullowa (53).

From observations published by Bullowa (53) and by Benjamin and his co-workers (55) it would appear that Type VIII pneumococcus pneumonia is a less serious disease than that due to other types. These investigators reported mortality rates of 12.7 per cent and 13.6 per cent respectively in relatively small series of untreated cases. On the other hand, Finland (57) noted a mortality of 34.8 per cent in 227 untreated cases. The numbers of serum-treated cases are as yet too small to permit making a definite statement as to the value of specific therapy, although the mortality appears to have been reduced slightly.

We have already commented on the great prevalence of Type XIV pneumococcus infections in infants and young children. While the results of serum therapy in relatively few cases have been reported, we have insufficient data to warrant particular comment. Further observations are required before an appraisal of serum therapy in this type of infection can be made.

It would appear that definite benefit can be expected from serum therapy in certain of the higher types of pneumococcal pneumonia, particularly Types V and VII. Further evidence, from large series of controlled cases, is urgently needed, however, to establish more exactly the value of this form of therapy in cases due to the higher types.

Rabbit Antipneumococcus Serum. Horsfall (58, 59) and his associates have reported on the use of unconcentrated antipneumococcus rabbit serum in the treatment of patients with pneumococcus pneumonia. A number of reasons have been advanced to indicate that it may be superior to antipneumococcus horse serum.

Unconcentrated antipneumococcus rabbit serum has a high mouse-protective value, being several times that of unconcentrated horse serum. Its protective value is at least equivalent to that of concentrated antipneumococcic horse serum.

There are certain differences in the immunologic activity of antipneumococcic rabbit serum as compared with that from the horse which

may be important in a consideration of the therapeutic advantages of each.

It had long been known that successful protection experiments in mice were dependent upon the use of optimal amounts of horse antiserum and culture, otherwise a zonal phenomenon might be encountered — the so-called *Schwellenwert* of Neufeld and Haendel. This phenomenon has been studied further by Goodner and Horsfall (60), who found that the prozone does not occur when antipneumococcus rabbit serum is employed. Goodner and Horsfall (61) have also shown that physiologic amounts of cholesterol and cephalin inhibit the mouse-protective action of antipneumococcus horse serum but do not affect the protective action of antipneumococcus rabbit serum.

An important difference between these two sera is in regard to the size of the respective antibodies. On the basis of ultrafiltration experiments reported by Elford, Grabar, and Fisher (62), and by Goodner, Horsfall, and Bauer (63) and experiments by the method of ultracentrifugation reported by Biscoe, Herčík, and Wyckoff (64) and by Heidelberger and Pedersen (65), it would appear that Type I antibody from horse serum is three to four times larger than Type I antibody from rabbit serum. It was pointed out that the penetration of rabbit antibody into infected tissues may be greater because of this smaller size. Observations by Horsfall *et al.* on infected pleural cavities would appear to support this opinion.

It is also pointed out that rabbits can be immunized much more quickly than horses and that the cost of production of serum is much less in the former animal than in the latter.

Antipneumococcus rabbit serum was produced by Horsfall and his associates in the following manner: Rabbits were given repeated intravenous injections of a formalinized suspension of pneumococci. When suitable antibody titers occurred, the animals were bled every two weeks by cardiac puncture, 50 cc. of blood being withdrawn at each bleeding. The serum was collected, pooled, filtered through a Berkefeld V candle, methiolate added to a concentration of 1 : 10,000, and stored in the ice-box. When a suitable quantity was obtained, the serum was heated to 56° C. for 30 minutes, cooled, and adsorbed with sterile, washed kaolin for 15 hours at 4° C. The kaolin was removed by centrifugation. The serum was again filtered through a Berkefeld V candle, filled into vials, and toxicity and sterility tests done.

When Horsfall and his associates first started to use rabbit antiserum, it was found that chill and thermal reactions were not uncommon. Treating the serum by adsorption with kaolin appeared to reduce the chill-producing substances. Furthermore, it was found that the simultaneous oral administration of a large dose of acetylsalicylic acid was of benefit in controlling thermal reactions. Others who have used anti-

pneumococcic rabbit serum intravenously have found thermal reactions to be rather frequent and on occasion alarming. For this reason, it is advisable that this product be used only in hospitals and by those experienced in serum therapy until more information is available concerning abnormal reactions following its use.

It is very important to explore the possibility of serum sensitivity before the use of rabbit antiserum. The usual history of allergic manifestations should be taken. The Rockefeller group have employed three sensitivity tests before the intravenous administration of serum. An *intradermal test* was done using 0.1 cc. of a 1 : 10 dilution of normal rabbit serum in physiologic salt solution. This test was found to be unsatisfactory in that all patients exhibited a reaction that could be interpreted as positive. In their group of cases none of these positive intradermal tests proved significant as indicating sensitivity to rabbit serum. A *conjunctival test* consisting of the instillation of one drop of a similar dilution of rabbit serum into one conjunctival sac was done. None of their patients gave a positive response to this test. Finally an *intravenous test* was done by injecting 0.1 cc. of the type-specific rabbit antiserum to be used for therapy, diluted with 5 cc. of physiologic salt solution. A positive response to this test consisted of a fall in arterial blood pressure and an increase in the pulse rate within five minutes after the injection. None of their patients exhibited a positive response to this test.

Horsfall and his associates have reported their experience in the treatment of 22 cases of lobar pneumonia with rabbit antiserum. This series consisted of Types I, II, VII, and VIII infections. The following table, taken from their paper, summarizes the results obtained in this group of cases.

TABLE III

TYPE-SPECIFIC ANTIPNEUMOCOCCUS RABBIT SERUM THERAPY IN PNEUMOCOCCIC PNEUMONIA

TYPE	CASES	BACTERIEMIA, NUMBER OF CASES	PLEURAL EXUDATE INFECTED, NUMBER OF CASES	FIRST SERUM AFTER ONSET, AVERAGE HOURS	CRISIS AFTER FIRST SERUM, AVERAGE HOURS	SERUM AVERAGE CC.	MORTALITY PER CENT
I	10	8	1	42	23	124	0
II	4	1	2	74	42	231	25
VII	3	1	0	33	19	145	0
VIII	5	2	0	38	23	173	0
Total	22	12	3	46	26	159	4.5

Taken from Horsfall, *et al.* (59).

A mortality of 4.5 per cent in such a small series of cases is, obviously, not significant. However, the clinical evidences of striking benefit in individual cases indicated that rabbit antiserum, in this series, was very effective. Crisis occurred with striking rapidity after the administration of serum, and signs of intoxication disappeared rapidly. Of considerable importance is the observation that empyema did not develop in two patients with grossly infected pleural cavities. Type-specific rabbit antibody was demonstrable in these exudates after the intravenous administration of serum.

The Treatment of Pneumonia with Vaccines. The developments of recent years have revealed certain immunological conditions in pneumococcus infection which may be considered as furnishing at least some basis for the use of specific vaccines in pneumonia, a matter which formerly rested on a purely empirical basis. Paramount among these is the fact that specific antibodies appear very early in the circulation of man and experimental animals following the injection of a suitable pneumococcal antigen, a situation in contrast to the slowness with which an animal produces antitoxin following the injection of bacterial toxins or toxoids. Barach (66, 67) demonstrated protective substances in the serum of rabbits three days after the administration of a killed vaccine of Types I and II pneumococci. The injection of filtrates of broth cultures occasioned a similar appearance of immune bodies on the fourth day.

Goodner (68, 69) made a detailed study of pneumococcal immunity in rabbits, in which it was possible to induce with virulent pneumococci a skin lesion that has close pathological and clinical analogies to human pneumonia. Among other things, the spontaneous appearance of antibodies and the effect of vaccines were studied in these animals. It was found that, as in lobar pneumonia, when occasionally animals recovered spontaneously, recovery was accompanied by the appearance of antibodies in the blood stream — an occurrence which, in human lobar pneumonia, takes place just before, during, or soon after the crisis. In rabbits a single injection of a Type I pneumococcus vaccine was followed with almost unvaried regularity by the appearance of antibodies on or about the fifth day after the vaccine injection. When rabbits were infected with an ordinarily fatal dose of organisms by the intracutaneous route five days after the vaccine injection, they developed practically no disease, either locally or systemically. When they were so infected three days after the vaccine injection, they ran a typical course for one day and the following day came down by crisis and recovered. If infected two days after the vaccine injection, they ran a course of three days and came down by crisis, again on the fifth day after the vaccine injection, at a time when the appearance of antibodies in the blood was to be expected. If

the vaccines were given after the infection, it is true, the course of the disease was not materially affected.

The question arises, Why should the additional administration of a moderate dose of vaccine assist in a process in which the same organism in large numbers is present in the patient's lungs and, to some extent, may be invading his circulation? This is, of course, the old objection to vaccine therapy in acute infectious diseases, and has a great deal of weight. On the other hand, it is not at all impossible that in pneumonia, at least, there is much to interfere with auto-immunization of the patient from his own lesion. Moreover, when vaccines or other modified substances are introduced into the subcutaneous tissues before circulatory invasion has occurred, they may aid against such an occurrence by stimulating the early appearance of antibodies.

Numerous reports have appeared on the use of vaccines in the treatment of pneumonia with results that can hardly be said to make an irrefutable case for this form of therapy. Lambert (70) presented observations with partially favorable results in a series of over 200 cases in which he had used a stock vaccine containing a number of organisms including pneumococci of several types, streptococci, staphylococci, *H. influenzae*, etc. Similar observations on the use of a vaccine produced from a mixture of a number of virulent strains of pneumococci were made by Wynn (71). The significance of results obtained following the use of such mixed vaccines can reasonably be doubted. Other reports which suggest some therapeutic value of vaccines have been published by Barach (72), and by Sutton, Kendall, and Rosenblum (73).

We feel that the value of the vaccine treatment of pneumonia has not been sufficiently established to justify recommending its continuance. Our position in this regard gains additional support from the excellent results which have been obtained by serum therapy.

The Prophylaxis of Pneumonia by Vaccines and Various Immunogenic Constituents of Pneumococcal Cells. Owing to the high death rate from pneumonia in mining camps, such as those in South Africa, and among soldiers in concentration camps during the War, it is natural that efforts should have been directed toward the development of methods for specific prophylaxis.

Probably the most extensive studies in this direction have been those in the gold fields and diamond mines in South Africa. Maynard (74) and Wright, Morgan, Colebrook, and Dodgson (75, 76) were among the first to employ vaccines in these regions. The extensive experience of Lister and Ordman (77) in South Africa has recently been reviewed in a monographic publication in which they report encouraging results. They have used a mixed vaccine consisting of several predominating types of pneumococci in addition to strains of streptococci, influenza

bacilli, *Staphylococcus aureus*, *M. catarrhalis*, and the Friedländer bacillus. Peall (78) reported his experience of several years in which 68,000 native workers were given three doses of a mixed vaccine at weekly intervals with a drop of 70 to 80 per cent in the incidence of pneumonia and other infections of the respiratory tract.

Cecil and Austin (79) and Cecil and Vaughan (80) made an extensive study of prophylactic pneumococcal vaccine in army camps during the war. At Camp Upton they vaccinated over 12,000 men against Types I, II, and III, administering three or four doses of a vaccine at intervals of 5 to 7 days, with a total dosage of six to nine billion of Types I and II, and four to six billion of Type III. Forty per cent of the personnel of the camp was vaccinated. Among the unvaccinated, there were 173 cases of pneumonia of all types, and among the 40 per cent vaccinated, there were 17 cases. A similar experience was carried out at Camp Wheeler, the only difference being that a lipovaccine, that is, a suspension of pneumococci in neutral fat, was used. Over 13,000, or 80 per cent of the men, were vaccinated. Here the period of post-vaccination observation was longer, that is, two to three months. During this period there were 32 cases among the 80 per cent vaccinated, and 42 among one fifth of the men who were unvaccinated.

Felton (81, 82, 83) has tested the immunity provoked by the injection of various water-soluble fractions of pneumococcal cells. He reported a fraction which lacked the property of precipitating immune serum but which immunized mice. A single two-milligram dose of this substance stimulated a degree of immunity which, as measured by protective antibodies in the serum, was comparable to that found in the serum of patients convalescent from pneumonia. Felton has reported two experiments in which large groups of young men were immunized with his soluble antigen. In the first of these, 3000 men received a single injection of 2 mg. each of Type I and Type II antigen. Nine thousand men living under the same conditions served as a control. During the period of observation there were eight cases of pneumonia with two deaths among the controls and no cases in the vaccinated group. In the second experiment 14,000 men were injected with 1 mg. each of the two antigens and an additional 14,000 men were uninoculated. There were 13 cases of pneumonia with no deaths among the inoculated men and 23 cases with 2 deaths in the control group.

Smillie (84) studied an epidemic of Type II pneumococcal pneumonia at the Veterans' Hospital at Bedford, Massachusetts. This outbreak had persisted for several months and was attended by a high mortality rate. Smillie vaccinated the entire population of the institution with a formalin-killed suspension of young, Type II pneumococci, following which the epidemic promptly ceased even though the carrier rate among

the inmates was not appreciably influenced. Smillie, Warnock, and White (85) also described an epidemic of Type I pneumococcal pneumonia at the State Hospital in Worcester, Massachusetts, in which 110 cases occurred during January and February, 1937. On March 2, 3, and 4 the hospital population consisting of 1900 individuals was immunized, each person receiving a single, subcutaneous injection of 2 mg. of Felton's soluble antigen. Nine cases of lobar pneumonia occurred between March 4 and June 1. Only 4 of these were due to the Type I pneumococcus, and three of them had not received the antigen. Unfortunately, in both these experiences, it was not possible, because of special circumstances, to vaccinate half of each population in order to control better the value of the prophylactic vaccination. It seems reasonable to conclude, however, that vaccination had at least some effect in bringing about a cessation of these outbreaks.

Duration of Pneumococcal Immunity. Many experimental observations such as those of Goodner with the intradermal infection of rabbits and those of Stillman and Goodner (86) indicate that pneumococcal immunity lasts a very short time, usually not more than a few months. The experimental evidence, moreover, gains support from certain epidemiological data. For example, Maynard found that the immunity conferred on native mine workers in South Africa by vaccination appeared to be effective not longer than four months. Furthermore, Finland and Winkler (87) have reported a series of cases of recurrent attacks of pneumonia. In some of these the second attack was due to the same type as that causing the first attack. The interval of time between attacks was short in a few of these.

The Production of Antipneumococcic Horse Serum. One of the most important considerations in the immunization of horses is the quality and characteristics of the immunizing antigen. It should, of course, be strictly type specific. It is now generally agreed that only cultures of maximal virulence furnish satisfactory immunizing material. Cultures of high virulence should be chosen and exalted virulence maintained by frequent passage through mice. At the Massachusetts State Antitoxin Laboratory the virulence of cultures is maintained by daily passage of strains through mice for five days a week.

In the past various efforts were made to employ different products of pneumococcal growth or autolysis as immunizing material. For example, filtrates containing soluble, cell-free components of the pneumococcal cell have been used. Such material has been shown to give rise to species-specific antibodies only, whereas immunization with intact pneumococcal cells gives rise to type-specific immunity. Exudates of infected pneumonic tissues have also been tried as immunizing material. It appears that the most satisfactory immunizing material consists

of whole, intact pneumococcal cells in which there is a natural combination of somatic protein and type-specific polysaccharide. Young cultures should be used for such a vaccine, and they should be prepared in such a manner as to halt spontaneous autolysis with the least possible physical or chemical trauma to the intact cells.

There has been much difference of opinion as to whether living or dead cells provide the most satisfactory immunizing material. For a long time the use of living cells held the ascendency; in such instances the horses were usually started with a series of injections of killed organisms with all subsequent injections consisting of living bacterial cells. There are many disadvantages to this method of great practical importance to the producing laboratory. A fresh culture must be prepared for each injection rather than preparing a bulk vaccine at one time sufficient for a series of injections. The greatest disadvantage is that there is a considerable mortality among the horses in spite of the most rigid precautions in the control of dosage. The crux of the problem is that there is no satisfactory evidence to indicate that a better therapeutic serum is produced by immunization with living cells, and the practice has been largely given up. Dead cells, if properly prepared, are equally satisfactory. Two methods have been commonly employed for killing pneumococcal suspensions, heat or treatment with formalin. Heat-killed preparations appear to be as satisfactory as formalin-killed and are much less toxic than the latter. At the Massachusetts State Antitoxin Laboratory pneumococcal suspensions are killed by heating at 100° C. for 15 minutes. Such suspensions have been adequate as antigenic material. Furthermore, it would appear that such suspensions resist autolysis much longer than those prepared by either formalin or heating at the usual lower temperatures (55° to 60° C.). It is desirable that all bacterial suspensions be properly standardized in order to secure relatively accurate and standard dosage. A satisfactory suspension is one containing approximately 25 billion organisms per cc.

Bacterial suspensions are generally administered by the intravenous route. At the Massachusetts State Laboratory (16) the schedule of injections with respect to spacing is as follows: The animals are injected on Tuesday, Wednesday, and Thursday of one week and again on Thursday, Friday, and Saturday of the following week. They are bled on Monday, the ninth day following the last injection. Injections of "holding doses," consisting of 2.0, 2.5, and 2.5 cc. of a suspension containing 25 billion organisms per cc., are resumed on the day following bleeding. Horses are, of course, started on small doses until the "holding doses" are attained. Individual animals may require some variation from this dosage, depending on their response.

Until recently it was common practice to produce polyvalent or bi-

valent sera. Patients received such sera on admission to the hospital, to be followed later by monovalent serum when the type of infecting organism had been ascertained. With the present method of rapid typing such a practice is not only unnecessary but undesirable. As a result monovalent sera are generally produced at the present time. There may be some justification for continuing to produce bivalent sera for closely related types such as Types II and V, for example.

Concentration of Antipneumococcic Serum. Numerous methods both biological and chemical have been proposed from time to time for the concentration of antipneumococcic serum. It is beyond the scope of the present text to describe or discuss all these methods. The procedure most commonly used at the present time is that developed by Felton (88), employing ethyl alcohol as a precipitant or some modification of this procedure. Briefly, this procedure is as follows: Ethyl alcohol (95 per cent) and the serum are chilled to 0° C., following which the alcohol is added to the serum in the proportion of 20 cc. of alcohol to 100 cc. of serum. The mixture is allowed to stand for 18 hours in the cold and the precipitate recovered by filtration or centrifugation. After washing the precipitate with one and one half volumes of chilled distilled water to remove the water-soluble protein, the water-insoluble fraction is dissolved in the desired volume of physiologic salt solution. The acid-insoluble fraction may be removed by suspending the alcoholic precipitate in water or by adding sodium chloride to effect solution and then adjusting the reaction to a *p*H of 5.0. The acid-insoluble precipitate which forms may be removed by centrifugation. It has been found that at least 80 per cent of the protective antibody in serum may be obtained by this method.

For details of the further processing of serum prior to its distribution for therapeutic use and for a detailed discussion of other methods for concentration the reader is referred to the monograph by White and his collaborators (16).

The Standardization of Antipneumococcic Serum. Numerous methods have been proposed for the estimation of antibody content or potency of antipneumococcic serum. These methods can be conveniently divided into three general classes.

A. Serological methods. Among the various serological methods may be mentioned agglutination, complement-fixation, and precipitation of antibody with purified type-specific polysaccharide. The latter method offers promise of more general adoption. A high degree of correlation may be obtained between results obtained by it and by *in vivo* protection experiments.

B. Therapeutic tests. Various therapeutic tests in experimentally infected animals have been employed from time to time. These depend on the administration of the test serum some hours after the injection of the infecting dose of organisms. These tests have been largely given up because of the many obvious variables that are difficult to control.

C. Mouse-protective tests. These tests involve the administration of the serum previous to or simultaneously with the injection of the infecting dose of culture. Felton (89), in 1924, defined the unit of antibody as that amount of serum which would protect mice against 0.5 cc. of a 1 : 10 dilution of an 18-hour broth culture of such virulence that 10^{-7} cc. killed mice in 36 to 48 hours. This dose represents one million minimal lethal doses.

Because of the many variables entering into the mouse-protective test it is impractical to be guided by a rigid definition. Though not ideal, it is obviously better to standardize serum against a known standard serum. Such a procedure is the one generally employed at the present time, the comparison of the unknown with the standard serum being made by mouse-protective tests.

The reader is again referred to the monograph of White (16) for a detailed description and discussion of the various standardization procedures and their limitations.

We include here a detailed description of the standardization of serum by the mouse-protective test. This procedure, which is a modification of the Park-Cooper (90) technique and that of the American Drug Manufacturers' Association, has been employed by the Massachusetts Antitoxin and Vaccine Laboratory for several years. Our purpose in giving it is that it affords a typical example of the biological assay of an antibacterial serum.

The following description is taken directly from the monograph by White (16).

"*a. Culture.* Virulence. The virulence of the cultures used should be such that no more than 3 to 10 organisms, as determined by 24-hour blood-agar plate counts, are required to kill at least two of three mice within 48 hours after intraperitoneal inoculation. Passages of the cultures through mice are made five days each week for maintenance of virulence.

"The culture used for a protection test is obtained in the following manner: A mouse is injected intraperitoneally with 0.5 cc. of an 18–hour broth culture; 6 hours later the mouse is chloroformed and a sample of the heart's blood is inoculated, by means of a sterile capillary pipette, into broth and on a blood-agar plate. After 48 hours' growth, if the cultures are pure, a transfer of 1.0 cc. of the broth culture is made to 15.0 cc. of broth and a drop of defibrinated normal horse blood added. This culture is allowed to grow for 4½ hours and is the one used in the test.

"(1) Virulence test: As a part of the mouse protection test, the virulence of the culture is checked by injecting three doses into mice in triplicate with 0.5 cc. of dilutions of the 4½ hour culture made in peptone solution. Fluctuations in the virulence and amount of growth of the culture from time to time may require variations in the dilutions, and those used in a given test are determined upon the basis of previous tests. The dilutions employed have thus varied from 1 : 100 million, 1 : 300 million, and 1 : 1000 million to 1 : 250 million, 1 : 750 million, and 1 : 2500 million for Type I; and 1 : 90 million, 1 : 270 million, and 1 : 900 million to 1 : 125 million, 1 : 375 mil-

lion, and 1 : 1250 million for Type II. Dilutions for other types are determined by experience. Virulence test dilutions are made in sterile test tubes.

"(2) Test dose of culture: The test dose of culture used in determining the protective power of serums is 0.5 cc. of a dilution of the 4½-hour culture made in peptone solution. The dilution employed depends upon fluctuations in growth and virulence (mentioned above) and must be determined by experience. For Type I tests, the dilution used has varied from 1 : 1000 to 1 : 2500; for Type II from 1 : 9000 to 1 : 12,500. The test dilution of culture is usually made in a sterile 125 cc. or 250 cc. Erlenmeyer flask, the amount depending upon the number of mice to be injected. A loopful of the test dilution is streaked on a blood-agar plate as a purity test and, in addition, three mice are injected with the test dose of culture alone.

"Illustrations of the method of making culture dilutions in typical tests, for a total of 132 mice, are shown in the following tables:

TYPE I

TUBE NO.	CC. OF CULTURE	CC. OF STERILE PEPTONE	DILUTION	CC. CONTAINED IN EACH 0.5 CC. OF ORIGINAL CULTURE
1	1	4	1 : 5	1×10^{-1}
2	1 of No. 1	9	1 : 50	1×10^{-2}
3	2 of No. 2	18	1 : 500	1×10^{-3}
4	15 of No. 3	60	1 : 2500 *	2×10^{-4}
5	1 of No. 4	9	1 : 25,000	2×10^{-5}
6	1 of No. 5	9	1 : 250,000	2×10^{-6}
7	1 of No. 6	9	1 : 2,500,000	2×10^{-7}
8	1 of No. 7	9	1 : 25,000,000	2×10^{-8}
9	1 of No. 8	9	1 : 250,000,000	2×10^{-9}
10	2 of No. 9	4	1 : 750,000,000	0.66×10^{-9}
11	1 of No. 9	9	1 : 2,500,000,000	0.2×10^{-9}

* This is the test dilution of culture.

TYPE II

TUBE NO.	CC. OF CULTURE	CC. OF STERILE PEPTONE	DILUTION	CC. CONTAINED IN EACH 0.5 CC. OF ORIGINAL CULTURE
1	1	9	1 : 10	5×10^{-2}
2	2 of No. 1	3	1 : 25	2×10^{-2}
3	1 of No. 2	9	1 : 250	2×10^{-3}
4	2 of No. 3	18	1 : 2500	2×10^{-4}
5	15 of No. 4	60	1 : 12,500 *	4×10^{-5}
6	1 of No. 5	9	1 : 125,000	4×10^{-6}
7	1 of No. 6	9	1 : 1,250,000	4×10^{-7}
8	1 of No. 7	9	1 : 12,500,000	4×10^{-8}
9	1 of No. 8	9	1 : 125,000,000	4×10^{-9}
10	2 of No. 9	4	1 : 375,000,000	1.33×10^{-9}
11	1 of No. 9	9	1 : 1,250,000,000	0.4×10^{-9}

* This is the test dilution of culture.

"***b. Serums.*** All dilutions of serums are made in physiological salt solution, the amounts of diluent being drawn from an accurately graduated burette. Accurately graduated pipettes are used for mixing dilutions, using a separate pipette for each mixture. Each dilution is drawn up into the pipette 25 times to ensure thorough mixing. Excess serum adhering to the outside of the pipette after removal from the original sample is removed with a pledget of cotton. Not less than 1.0 cc. of a given serum dilution is used for making any higher dilution, and the volume of each dilution is kept as small as possible (usually 15 cc. or less) so that mixing may be thorough. (In making the first dilution of the control serum only 0.5 cc. of serum is used in order to conserve it.) The test dose of all serum dilutions is contained in a volume of 0.5 cc.

"(1) Control Serum: The control serum is one supplied by the National Institute of Health and is, at present, P 11, to which have been assigned unit values of 300 and 150 units per cubic centimeter for Types I and II respectively. The dilutions used are for Type I, 1 : 1000, 1 : 2000, and 1 : 4000; for Type II, 1 : 250, 1 : 2500, and 1 : 5000. These dilutions are made in the following manner:

TYPE I

0.5 cc. serum + 4.5 cc. saline = 1 : 10 (*a*)
1.0 cc. of (*a*) + 9 cc. saline = 1 : 100 (*b*)
2.0 cc. of (*b*) + 8 cc. saline = 1 : 500 (*c*)
8.0 cc. of (*c*) + 8 cc. saline = 1 : 1000 (*d*)
4.0 cc. of (*d*) + 4 cc. saline = 1 : 2000 (*e*)
2.0 cc. of (*d*) + 6 cc. saline = 1 : 4000 (*f*)

TYPE II

(*a*), (*b*), and (*c*) dilutions as above
6.0 cc. of (*c*) + 9 cc. saline = 1 : 250 (*d*)
4.0 cc. of (*d*) + 4 cc. saline = 1 : 2500 (*e*)
2.0 cc. of (*d*) + 6 cc. saline = 1 : 5000 (*f*)

"In each case, only the (*d*), (*e*), and (*f*) dilutions are used for injecting mice.

"(2) Unknown Serums: The dilutions used for injecting mice must bear the same ratio to each other as dilutions (*d*), (*e*), and (*f*) of the control serum. As concentrated serums are ordinarily much stronger than the control, obviously the dilutions will be much higher. For example, if a serum being tested for Type I antibody is assumed to be about 10 times as strong as the control serum, the dilutions used for injecting mice will be 1:10,000, 1:20,000, and 1:40,000, these being reached by making dilutions in steps similar to those for the control.

"***c. The test.*** At least two complete tests giving consistent results and preferably made on different days, using 10 mice for each dilution of serum, must be made for final estimation of the potency of an unknown serum.

"(1) The Mice: It is preferable to use mice of a highly inbred, uniformly susceptible stock, in sound, healthy condition. The weights should lie between 16 and 20 grams each.

"(2) Method of Injection: Serum and culture dilutions are not mixed prior to injection. Two 2 cc. vaccine syringes, fitted with 26 gauge, $\frac{3}{8}$ inch Schick needles, are used for injecting the culture. Two ordinary 2 cc.

hypodermic syringes, fitted with needles as above, are used for each serum. One operator fills the syringes, a second makes the injections, and an assistant handles the mice. The serums are always injected first, starting with the highest dilution, and followed immediately by the test dose of culture. Any mice improperly injected are discarded and replaced by others. Four serums (including the standard) and the requisite controls require 132 mice. From 30 to 40 minutes are required for injecting this number.

"(3) Length of Test: Observations are made at least twice each day and the approximate hour of death is recorded for mice found dead. The test is terminated at the end of 96 hours and all mice living at this time are recorded as survivals.

"(4) Necropsies: Necropsies should be performed on a sufficient number of mice to prove death due to uncontaminated pneumococcus infection. Streaking a sample of the heart's blood from a sterile capillary pipette on a blood-agar plate is adequate.

"***d. Interpretation of the test.*** In interpreting the test, no attempt is made to identify an end-point dilution, because such a procedure is statistically unsound inasmuch as it considers the fate of too small a group (10 mice). As the same number of mice is used for each serum and as the doses used for each serum are in the same ratio to each other, it is permissible to take into account the whole number (30) injected with each serum. The method of computation of potency values is, therefore, based on the equation:

$$\text{Units per cc. in unknown serum} = \frac{x}{y}\left(a + \frac{a\,(s - s')}{n}\right)$$

x = largest amount of control serum injected
y = largest amount of unknown serum injected
a = units per cc. in control serum
s = number of mice surviving on unknown serum
s' = number of mice surviving on control serum, and
n = total number of mice injected with all dilutions of any one serum (see above).

"If s is less than s', then the part of the formula within the inner parentheses has a minus value and is subtracted from a, not added to it. The value of n will be 30, 60, 90, etc., depending upon the number of tests from which calculations are made."

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CHAPTER XXVI

TYPHOID FEVER AND OTHER ACUTE ENTERIC INFECTIONS

Typhoid Fever

Immunity in Typhoid Fever. Typhoid fever offers many difficult immunological problems. The organisms get into the body through the intestinal mucosa, where at first they accumulate in the course of the incubation time until, at or about the end of the tenth or twelfth day, they have penetrated into the lymphatics through an inflamed mucous membrane, have produced local inflammatory lesions in the solitary lymph follicles and Peyer's patches and penetrated into the blood stream. During the first ten or twelve days thereafter the disease is in part a bacteriemia, the organisms circulating in the blood, probably washed out of the localized lesions in the intestinal wall. Antibodies are found early in the disease, and it is well known that between the end of the first and the middle of the third week, agglutinins and bactericidal sensitizers can be demonstrated in most cases and, in some cases, in considerable concentration. Nevertheless, the disease progresses and may continue for several weeks and end with death in spite of a considerable serum antibody content. Moreover, a patient may exhibit a typhoidal relapse early in the convalescent period during which the entire course of the disease may be repeated with all the usual manifestations, including a positive blood culture and with a high concentration of antibacterial antibody being demonstrable throughout this entire period. We have had the opportunity to observe such a patient in whom a high serum bactericidal power was demonstrated during the latter part of the initial disease, during the convalescent period, and throughout the relapse (1). It is difficult to account for situations of this character.

In the usual course of the disease it is probable that the typhoid bacilli disappear from the blood stream in the second and third week as a direct result of these antibody concentrations and that thereafter they remain lodged in tissues where they are protected from serum injury. This fact of the capacity of various microorganisms to maintain themselves in certain tissues when they are destroyed in the blood stream is well known and common in bacterial infection. The persistence of organisms in the spleen when they have disappeared from the blood has been observed

with many bacteria, and Hopkins and Parker (2), some years ago, showed that streptococci would persist in muscle tissues when they were being actively destroyed in lung, liver, spleen, and blood stream. The most commonly observed instance is the persistence of streptococcus viridans in heart valves, continuously being discharged thence into the blood stream, when the opsonic capacity of the circulating blood is higher than normal.

In typhoid and paratyphoid fevers what probably occurs is that after the development of antibodies the organisms are kept out of the blood stream but persist in the spleen and in lymph nodes, especially of the intestinal wall, and thence liberate the toxic principles which continue the disease. When cure is effected, it probably means an additional development of tissue immunity and increased capacity for bacterial destruction on the part of fixed cells, a mechanism which as far as it is at all understood has been discussed in the chapters on phagocytosis.

Another possibility that merits consideration in this regard is that typhoid bacilli may remain viable after being phagocytized and be protected from humoral antibody in such an intracellular location. Rous and Jones (3), in 1916, showed experimentally that phagocytized typhoid bacilli were protected from antibody and from potassium cyanide. They concluded that "Living phagocytes are able to protect ingested organisms from the action of destructive substances in the surrounding fluid, and even from a strong homologous antiserum." Moreover, Fothergill, Chandler, and Dingle (4) have described evidence suggesting that *H. influenzae* may be protected from the destructive action of antiserum when ingested by phagocytes and that this may be a factor in the frequent failure of antiserum in the treatment of patients with *H. influenzae* meningitis.

The so-called "permanent" immunity following typhoid fever, as well as such diseases as cholera, plague, and others, is possibly not as solid as generally supposed. If one considers that only a small fraction of a population contracts typhoid fever in ordinary times, and that the fraction of this fraction which is exposed would constitute an extremely small percentage of recurrences, and if one considers, in addition, that as people grow out of the early adult years which have the greatest typhoid incidence, the acquisition of regularity in habits of life considerably reduces their exposure, one is prone to question whether our information concerning permanent immunity is accurate.

In spite of all this, however, it is hardly questionable that a certain amount of increased resistance persists for a long time and long after antibodies have disappeared from the blood. This probably means that the tissues of a recovered individual are immunologically extremely sensitive in their reactions to typhoid antigen, and that when, reinfected,

they come in contact with the first small amounts of typhoid antigen, they respond with antibody production far exceeding in speed and amount that of a normal person.

Prophylactic Immunization in Typhoid and Paratyphoid Fever. The first attempt to inoculate human beings with typhoid bacilli with the intention of producing prophylactic immunization was that made by Pfeiffer and Kolle (5) in 1896. Wright (6) made similar studies in England, and reported upon the development of antibodies in the blood of 17 people inoculated with killed typhoid bacilli. By these studies it was shown that human beings could be inoculated in this manner without danger, and this logically led to the attempt to vaccinate on a large scale.

It is hardly necessary to dwell upon the desirability of such a procedure. From tables published by Russell (7) it appears that, in our own Spanish American war, 20,738 cases of typhoid with 1580 deaths occurred in a total enlistment of 107,973. In this war, 243 men were killed in action or died of their wounds, while almost seven times as many died of typhoid fever. In the British army during the Boer war there were over 75,000 cases of typhoid in 380,000 men, and in the Russian army during the Russo-Japanese war over 17,000 cases of typhoid occurred, over half as many as the number of men killed in action. Such appalling figures leave no possible doubt as to the desirability of prophylactic immunization in armies, and there can be little question that typhoid fever is sufficiently prevalent in many parts of the civilized world to encourage prophylactic immunization of individuals under conditions which render exposure likely.

Following the preliminary studies of Pfeiffer and Kolle and of Wright, practical studies were made in the German colonial army during the Herrero war, and by British bacteriologists during the Boer war. Leishmann (8) also studied the results of vaccination among regiments of the British army in India.

The vaccine employed by Wright and his associates in England consisted of broth cultures of a typhoid bacillus killed by heating and by the addition of 0.4 per cent phenol. The German vaccine consisted of emulsified agar cultures similarly killed.

The results obtained with these vaccines, although encouraging, were not as striking as had been hoped. Russell, summarizing the earlier attempts, stated that the morbidity was reduced to about one half among vaccinated persons with a slightly greater reduction of mortality. He attributed the early failures to the overheating of the vaccines, with consequent reduction of their antigenic properties, and to timidity in their administration resulting from Wright's fear of a negative phase. Russell produced his vaccines as follows: A single strain of typhoid bacillus was used (culture Rawlings obtained from Wright), and this was

grown on agar in Kolle flasks for 18 hours. The purity of the culture was tested both morphologically and by transplantation upon the double sugar medium devised by Russell. Agglutination tests were also made. After 18 hours the growth was washed off in small quantities of salt solution and the emulsion heated in a water bath for one hour at 53° C.; it was then diluted with sterile salt solution to a concentration of one billion bacteria to a cubic centimeter. Then 0.25 per cent of tricresol was added. Before use the safety of the vaccine was ascertained both by aerobic and anaerobic cultivation and by the injection into mice and guinea pigs of considerable quantities for the exclusion of possible tetanus contamination. The antigenic effectiveness of the vaccine was then tested by injecting rabbits with three doses at 10-day intervals, and determining the resulting agglutinating titer.

The troops mobilized in Texas, California, and along the Mexican border were treated with these vaccines under the direction of the United States Army Medical Corps. Compulsory vaccination was established in March, 1911, and the results as reported by Russell have fully justified the measure. The following table taken from Russell's paper illustrates the results obtained.

TYPHOID FEVER, OFFICERS AND ENLISTED MEN, UNITED STATES ARMY

	Year	Jan.	Feb.	Mar.	Apr.	May	June	July	Aug.	Sept.	Oct.	Nov.	Dec	Totals for 9 Mos.
Voluntary	1908	5	6	4	2	3	11	14	31	25	26	12	8	101
	1909	4	10	6	4	11	15	26	14	16	45	20	6	106
Compulsory	1910	8	11	1	4	2	6	12	27	21	16	20	11	92
	1911	3	3	3	7	4	4	4	7	4	4	1	0	39
	1912	1	2	2	0	0	3	1	3	1	4	0	1	13
	1913	0	0	0	0	0	0	0	0	0	0	0	0	0

Paratyphoid fever included in figures for 1908, but excluded in other years. Cases paratyphoid 1909, 3; 1910, 3; 1911, 2; 1912, 3; 1913, 0.

Taken from Russell (7).

Other methods for immunization against typhoid fever were proposed from time to time. Metchnikoff and Besredka (9), in their studies on typhoid vaccination in the chimpanzee, concluded that vaccination with dead typhoid vaccines was of little value, whereas animals vaccinated with small amounts of living cultures were protected. Metchnikoff and Besredka adopted, in consequence, the method of immunizing with living sensitized vaccines. By this is meant typhoid bacilli that have been exposed to the action of heated immune serum, and have absorbed

specific antibodies. There is no question as to the efficiency of this form of vaccination. However, the results of Russell and others have demonstrated that in human beings the use of dead vaccines is of considerable value, and there are certain practical objections to the use of living vaccines in immunization of large numbers of people. Living vaccines cannot be stored for any considerable period, and may become a source of possible infection by mouth if carelessly handled; furthermore, contamination is not so easily ruled out in the case of living vaccines when used on a large scale.

Besredka (10) has vaccinated against the disease by the oral administration of dead typhoid bacilli mixed with ox bile to produce, as he believed, a local immunity in the small intestine. This method has been studied in this country by Hoffstadt and Thompson (11, 12, 13) in particular. While large numbers of people have been treated by this method, the available evidence is not sufficient to justify the conclusion that it is an effective method of immunization.

The method used in the German, British, and American armies, *i.e.*, vaccination with dead cultures, seems certainly, according to Russell's statistical studies, to have yielded excellent results and recommends itself by its extreme simplicity and safety. The proof of this became conclusive during the last war when armies were free from typhoid fever, in spite of the most unfavorable sanitary conditions existing during prolonged battles in areas of concentration. According to Castellani (14), there were only 458 cases of typhoid and paratyphoid fever among 500,000 white Italian troops in Africa during the recent Italo-Ethiopian war. This number of cases was said to be no greater than among a similar number of troops at home during the same period. While scrupulous attention was given to water and food supplies and to personal hygiene, nevertheless, a good deal of credit for such a remarkable freedom from typhoid fever during this campaign in a tropical country can be credited to prophylactic vaccination. In this instance a so-called tetra vaccine was used, *i.e.*, a mixed vaccine for immunization against typhoid, paratyphosus A, paratyphosus B, and cholera.

It is evident, therefore, that the methods first recommended by Russell and others have stood the actual test on many millions of men. Nevertheless, with a rapid accumulation of knowledge of bacterial dissociation and antigenic structure during recent years, it has become obvious that a great deal of attention should be paid to the choice of strains for vaccine production.

For some years the British and American vaccines have been, almost without exception, made with the original "Rawlings" strain of Wright. Investigations of recent years on bacterial dissociation, however, have revealed conditions which make it important to examine strains of bac-

teria used for vaccine production in order to determine whether they have retained the most favorable antigenic composition. With motile, Gram-negative organisms, the studies of Arkwright (15, 16), Gardner (17), Li (18, 19), and others have revealed that, in this group, four possible mutation forms can develop, as follows:

1. A smooth motile, retaining flagellae.
2. A rough motile, retaining flagellae but probably lacking certain somatic antigenic material.
3. A smooth non-motile.
4. A rough non-motile.

Grinnell (20, 21), studying these mutation forms of the typhoid bacillus, has found conditions which do not correspond with the results obtained by Li with Salmonella suispestifer, but he has, nevertheless, determined considerable differences in the immunizing values of different mutation forms of this organism. Using bactericidal tests with whole blood, as a test for successful immunization, and comparing this with agglutination, Grinnell found that a vaccine made from a rough strain of *B. typhosus* "Rawlings" produced little increase in the bactericidal power of the blood for virulent organisms. Similar vaccination with a smooth, virulent typhoid bacillus produced a considerable increase in the bactericidal antibodies. Since vaccination with the rough strains leads to production of agglutinins for the virulent strains, but without increase in bactericidal power, he concluded that agglutination is not an adequate test of the resistance of an individual and that in so far as bactericidal power is more directly an index of resistance, typhoid vaccination with rough strains is relatively without prophylactic value. Grinnell made a further contribution that has been of great importance in subsequent studies of this problem. He developed a mouse-protective test that has been of great value in studying both the immunogenic qualities of various strains and the antibody response in vaccinated human beings.

During the past few years there has been considerable advance in our knowledge of the antigenic composition of the typhoid bacillus which has a great practical bearing. Topley and Raistrick in England and Boivin in France have isolated and studied the somatic or O antigen, which appears to be a combination of a lipoid and polysaccharide. This substance is antigenic and toxic. It undoubtedly represents the so-called endotoxin of the typhoid bacillus. It is a component of smooth, virulent strains and, as such, is an essential constituent of any strain to be used for prophylactic vaccination. The O antigen has not been isolated from completely rough strains. A more detailed discussion of this antigen and of the literature bearing on it will be found in the chapters on Antigens and Bacterial Toxins.

In 1934 Felix and Pitt (22, 23) described a new antigen in the typhoid

bacillus which they called the "Vi" antigen because of its presence in freshly isolated, fully virulent cultures. The existence of such a component was suggested by the fact that freshly isolated, living organisms are inagglutinable by an anti O serum. This antigen is very heat labile, heat-killed organisms being no longer inagglutinable. It is apparently a surface structure because its presence renders the O antigen resistant to the O antibody. A Vi antibody was demonstrated by agglutination and absorption tests and the *in vitro* titers of this antibody appeared to be rather low. It was shown that the Vi antibody had a powerful protective action in active and passive immunity experiments. The H and O antigens are unaffected by Vi antibody, as a result of which it is incapable of neutralizing endotoxin.

Felix (24, 25) and his associates in further studies of this antigen have described certain properties which are difficult of interpretation. They have shown that antisera of equal ability to agglutinate Vi strains can be produced by immunization with either living "natural" Vi antigen or formolized Vi antigen. The latter antiserum, however, was much inferior in phagocytosis-promoting activity and in protective power.

The extreme sensitivity of the Vi antigen to heat and to certain chemical agents would appear to challenge the usefulness of typhoid vaccines as ordinarily prepared, *i.e.*, killing the bacterial suspensions by heating at 56° C. Fortunately, such a gloomy prospect has not been supported in a number of extensive experiments in which the value of various preparations of vaccines has been tested by the mouse-protective technique. Schütze (26) compared the protective value of vaccines prepared from Vi strains after sterilization by a number of methods and found them to be essentially similar.

The extensive studies of Perry (27, 28, 29) and his associates in England and Siler (30, 31) and his co-workers in this country, both of whom employed the mouse-protective test as developed by Grinnell, demonstrated two important principles. Killing suspensions of typhoid bacilli by heat does not destroy their protective power. Of immense practical importance has been the demonstration, in confirmation of Grinnell's work, that vaccines prepared from rough strains are of little or no protective power. For adequate protection, typhoid vaccines should be prepared from fully virulent, smooth strains possessing a complete antigenic composition. The "Rawlings" strain has been given up by the Army Medical School in Washington. At the present time American troops are being immunized with a vaccine prepared from a strain "No. 58" which was isolated from a carrier in Panama. This strain is highly virulent and possesses good immunogenic qualities. Holt and Hitchens (32) have described the details of preparation of this vaccine.

At the present time it is common practice to employ a so-called "triple" vaccine, consisting of a suspension of killed *E. typhosus*, *S. paratyphosus* A, and *S. paratyphosus* B. Vaccines are standardized to contain 1000 million typhoid bacilli and 750 million of each of the paratyphoid organisms per cubic centimeter. Three subcutaneous doses are given at 7- to 10-day intervals, the first one of 0.5 cc. and the second and third of 1.0 cc. each. The dosage may be reduced in small children.

With regard to the paratyphoid organisms it is also important to select strains of complete antigenic composition.

In administering vaccine it is extremely important not to inject the preparation into a small vein. Such an accident may cause a serious chill and thermal reaction. Such an accident can be avoided by gently drawing back on the plunger of the syringe after the needle has been inserted. If no blood is aspirated it is unlikely that the tip of the needle is in a blood vessel. The vaccine should always be injected subcutaneously.

There is always a local reaction which may attain an area of 5 or 6 centimeters in diameter over which there is redness, swelling, and some pain. There may be swelling of regional lymph nodes. Suppuration should not occur in properly performed vaccination. General reactions vary in different individuals. In most healthy adults the first injection gives little trouble. But after the second or third there may be headache, nausea, and temperature, coming on several hours after the injection. In some cases the symptoms may persist for several days, and the patient feel very miserable, but no severe harm is done.

The indications for the use of typhoid vaccine are, of course, familiar to all. Any individual should be vaccinated who is apt to encounter circumstances which create a risk of typhoid infection even though that risk may be slight. All military personnel and any other group of individuals living a mobile existence because of special circumstances (crews aboard ship, etc.) should be vaccinated. Populations which have been stricken by some catastrophe such as flood, famine, etc., should receive typhoid inoculations. Individuals who are obliged to travel in remote regions where hygienic conditions are bad should be similarly protected.

A question of frequent concern to private practitioners and to public health officials is whether or not the household and other contacts of a case of typhoid fever should be vaccinated. Ramsey (33) has presented a detailed statistical analysis of a study of this question in New York State. He concluded that a reduction of 75 per cent from anticipated secondary cases can be expected among contacts who receive three doses of vaccine subsequent to the onset of the primary case. From this it would appear advisable to vaccinate all contacts, particularly in the same household, of a case of typhoid fever.

Therapeutic Vaccine Treatment in Typhoid Fever. From time to time various attempts have been made to treat typhoid fever patients with injections of typhoid vaccine. It is very likely that the beneficial results claimed by some have been due to non-specific foreign protein shock, since similar results have been reported following the injection of vaccines prepared from colon bacilli and from paratyphoid bacilli. From our present knowledge of the antigenic composition of typhoid bacilli it would appear that the organism, as it is present in the body of the patient, is in its most effective antigenic state as far as the stimulation of the production of bactericidal antibodies is concerned. In our opinion, the use of vaccine in the treatment of the active stages of the disease is not justified.

The Serum Treatment of Typhoid Fever. The first attempts to treat typhoid fever by passive immunization with the serum of immunized animals were made by Chantemesse (34, 35), who injected horses with filtrates of typhoid cultures subcutaneously and with emulsions of virulent bacilli intravenously. Chantemesse believed that such serum not only possessed bactericidal action, but stimulated phagocytosis, and possessed a certain limited amount of neutralizing power against the toxic properties of the typhoid filtrates. At the International Congress of Hygiene in Berlin in 1907 Chantemesse reported upon a thousand cases treated with his serum. Of this number only 43 died, whereas the average mortality during the same six years at the Paris hospitals was 17 per cent. The injection of the serum, he claimed, improved the condition of patients in that, after a preliminary period of from several hours to five or six days, the temperature declined and the general condition of the patient changed for the better. A remarkable feature of Chantemesse's treatment is that he injected into the patients a few drops only of the serum, and rarely made a second injection, a fact which alone tends to persuade one that his apparent therapeutic success was a fortunate accident.

Besredka (36, 37) prepared a serum by the intravenous injection of typhoid cultures heated to 60° C., continuing the immunization for six months. He claimed that this serum possessed what he designated as "anti-endotoxic" properties. A dried extract of typhoid bacilli which in a dose of 0.01 gram killed a guinea pig of 300 grams regularly, became innocuous when mixed with small quantities of this horse serum. One cubic centimeter of the serum often neutralized as much as two fatal doses of the extract, but Besredka stated particularly that even an increase of the quantity of serum never neutralized more than two fatal doses. This fact alone would militate against considering the serum of Besredka antitoxic in the sense in which the word is used concerning diphtheria and tetanus antitoxins. Besredka's anti-endotoxic serum was investigated

by Pfeiffer and Bessau (38), who found that it exerted a beneficial influence upon typhoid infection in guinea pigs if injected at the same time with the bacilli. It also protected somewhat against the toxic properties of substances derived from the typhoid bacillus, but Pfeiffer and Bessau did not believe that this was due to a true antitoxic action. Kraus and Stenitzer (39, 40, 41) took up the study of typhoid immunization from the point of view that the typhoid bacillus produces a true toxin, and that therefore a true antitoxic action could be expected from the sera produced by immunization with typhoid filtrates. A similar point of view was held by Arima (42). Kraus and Stenitzer (43, 44) later immunized horses and goats with extracts of agar cultures and with broth filtrates. The serum which they produced in this way not only possessed the ordinary bactericidal action, but, they claimed, neutralized toxic broth filtrates, not only of the typhoid, but of the paratyphoid bacilli. The serum of Kraus and Stenitzer was used by a number of observers, among them Herz (45), and the results were said to be encouraging in early cases. Rodet and Lagriffoul (46) immunized horses with living typhoid cultures and also claimed favorable results from the use of their serum.

Matthes (47), continuing the work of Gottstein after the death of the latter, employed the method of immunizing with the product obtained by digesting typhoid bacilli with trypsin. He spoke of the poison so produced as "fermotoxin." Lüdke (48) slightly modified the Gottstein-Matthes method by digesting the typhoid bacilli with pepsin and hydrochloric acid, and with the poison so produced immunized 8 goats, reinforcing the immunization by the subsequent injection of the bacilli themselves. He claimed that 0.05 to 0.1 cc. of the serum so produced protected animals against five times the lethal dose of the poison. He reported good results in a small series of human cases treated with this serum.

Garbat and Meyer (49) claimed that the most potent therapeutic serum could be obtained by the combination of sera produced by the injection of sensitized and of unsensitized bacteria. They asserted that the typhoid bacillus contained two definite antigens, one particularly associated with the bacterial ectoplasm, which becomes active when the bacteria enter the animal body, and a truly endocellular poison which does not become active until the surrounding ectoplasm is dissolved. They believed that sensitizing bacteria was a method for the production of endotoxin and thought that therefore the ideal serum for the treatment of typhoid consisted of a mixture of two sera produced each with one of the antigens, that is, with sensitized and unsensitized bacteria. Rommel and Herman (50) did not obtain encouraging results with this serum. The theory of Garbat and Meyer of two distinct antigens in the typhoid bacillus is of unusual interest in the light of our present knowledge of the

antigenic anatomy of the typhoid bacillus. Their theory, at least, foreshadowed the demonstration of the existence of the Vi and O antigens, although their experimental evidence does not warrant this conclusion.

From the great variety of different methods that were employed for producing an anti-typhoid serum in these older experiments it is obvious that no one of them was particularly successful in the treatment of the disease. A study of the literature of this period, as reviewed above, leaves one unconvinced as to the value of these products. The practical test of their value has been the fact that they were all gradually given up.

During recent years, however, with the development of knowledge of the antigenic components of the typhoid bacillus and the function of these substances in the production of disease, there has been a rebirth of interest in the possibilities of development of an effective therapeutic serum. For example, it has been postulated that a serum with a high O antibody content should be useful in relieving the toxic symptoms of the disease as well as exerting a bactericidal effect. It has likewise been thought that a serum containing a high Vi antibody content should be effective for the destruction of the organism and that definite therapeutic benefit should be expected from sera containing both antibodies. Sera possessing these qualities have been put to clinical trial by a number of investigators during the past few years.

Felix (51) carried out a clinical trial with his new serum in Palestine. A small number of control cases received normal horse serum. The therapeutic serum contained a high titer of both Vi and O antibody. The results of this trial were promising, although the number of cases treated was too small to warrant drawing definite conclusions. It was noted that there was a striking relief of toxemic symptoms in a number of patients, which indicated that the O antibody was not without some beneficial effect. During the same year McSweeney (52) treated 8 cases with a serum containing both Vi and O antibody. The dose of serum varied from 10 cc. to 300 cc. and was administered by intramuscular injection. This author felt that the most striking clinical effect was on the toxemia.

Robertson and Yu (53) have reported their experiences in the serum treatment of Chinese patients in Shanghai. Strains of high virulence and relative inagglutinability by O antibody were used for immunizing horses by methods similar to those of Felix. Fifty-two patients were treated with their serum, in doses of 50 cc. of unconcentrated and 25 cc. of the concentrated product. The serum was administered by intramuscular injection. Thirty of these cases showed definite improvement, as indicated by a drop in temperature and a decrease in toxic symptoms. Seven showed reduction in toxemia only and in 15 cases no appreciable effect from the serum was observed. Four additional cases were treated

with Felix's Lister Institute serum and two of these showed dramatic improvement. They felt that this new typhoid serum was definitely beneficial when given during the first or second week of illness in lessening the toxemia and shortening the febrile course.

Cookson and Facey (54) had an opportunity to treat 73 patients with Felix's serum during the course of a milk-borne epidemic of typhoid fever which involved some 500 persons. The dosage of serum averaged 24 cc. in children and 47 cc. in adults. Of these cases there was improvement from toxemia in 54 and in temperature in 40. Improvement occurred in 73 per cent of the cases within 48 hours of the injection of the serum. The mortality was 9.5 per cent, which, of course, is not striking but may, in part, be due to the fact that serum was given to the more severe cases.

While the data presented in these studies are not sufficiently large to justify expressing a definite opinion concerning the value of this new type of serum, they nevertheless are encouraging enough to warrant continued trial.

Bacillary Dysentery

Bacillary dysentery is an acute infectious disease involving the mucous membrane of the colon and, in a small percentage of cases, the lower portion of the ileum. It is usually of sudden onset and is characterized by fever, prostration, diffuse abdominal pain, and occasionally vomiting soon followed by diarrhea. As the mucosa of the bowel becomes ulcerated the diarrheal stools contain variable quantities of blood, pus, and mucus, findings which are typical of the disease. It should never be forgotten, however, that in mild cases, particularly in mild cases infected with Sonné's bacillus, the stools may not contain these characteristic elements. Recognition of this fact is important from the point of view of prompt isolation and control.

Bacillary dysentery and typhoid fever present many epidemiological features in common. In both diseases, the infectious agents are carried from the excreta of one individual to the mouth of another by various intermediate vectors such as food, water, milk, flies, fingers, etc. This would suggest that the same public health measures should be equally effective in the control of the two diseases. Such is not the case, however. Bacillary dysentery is not uncommon in communities where typhoid fever has been almost entirely eradicated. Furthermore, dysentery continues to be a vexatious problem in asylums, orphanages, prisons, and among concentrated military personnel.

There are a number of reasons to explain the continued presence of dysentery. Many cases are so mild as not to become bedridden, exhibiting little more than a mild "gastro-intestinal upset." Such individuals, if they are engaged in handling food, are a considerable source of danger.

While permanent carriers of dysentery baciili are extremely rare, nevertheless, occasional individuals discharge organisms for weeks or several months after convalescence.

There is an important difference in the pathogenesis of bacillary dysentery as compared to typhoid fever which has an important bearing on the control of the disease as well as on the applicability of diagnostic laboratory procedures. In dysentery the organisms multiply in the large bowel and the lesions of the mucosa are produced by direct contact of the organisms and their toxins with the mucosa. The deeper tissues and blood stream are rarely invaded. Blood cultures and urine cultures are therefore of no value as diagnostic procedures. Organisms are present in the stools from the very onset of symptoms, and, as a consequence, patients are infectious before they may have become bedridden. Moreover, stool cultures, taken early in the course of the disease, are the most logical procedures in laboratory diagnosis.

There are a number of types of dysentery bacilli differentiable by both serologic and cultural procedures, a matter of some importance in a consideration of procedures for specific prophylaxis and treatment. For practical purposes it is convenient to divide the dysentery bacilli into three main groups. The first of these is non-mannite-fermenting organisms and consists of the Shiga and the Schmitz bacillus. All other dysentery bacilli ferment mannite. The second group may be considered to be those organisms which ferment mannite but not lactose and consists of those strains usually described as the Flexner bacillus, the Hiss-Russell bacillus, and the Strong bacillus. As shown by Murray (55), this group can be divided into at least five serologic types. There is, however, a broad antigenic overlapping among them. The third group is composed of mannite-fermenting organisms which also ferment lactose after a variable incubation period and is typified by the Sonné bacillus and the Dispar bacillus. The latter is of little if any pathogenic importance.

Toxins of Dysentery Bacilli. It was early recognized that filtrates of broth cultures of *B. Shigae* were very toxic for rabbits following intravenous injection. It was likewise recognized by the early investigators that the toxin of this organism, which was considered to be an exotoxin, produced two types of lesions in rabbits, one involving the mucous membrane of the colon, the other confined to the central nervous system and presenting the histological picture of an acute encephalitis. Olitsky and Kligler (56) finally isolated two toxins from cultures of this organism and demonstrated that they were physically and biologically distinct. They showed that filtrates of several days' growth of the organism in egg albumin broth contained a substance that was destroyed by heating at 75° C. for 1 hour. It produced encephalitic lesions in rabbits but did not

affect the mucosa of the bowel. This substance was regarded as an exotoxin and a neurotoxin. The substance responsible for lesions in the intestine can be obtained by autolysis or disintegration of the bacillary cells and is of the nature of an endotoxin. It is relatively more heat stable than the exotoxin. Practically all investigators are agreed that the exotoxin is neutralized by antitoxin according to the laws of multiple proportions.

Some investigators have expressed doubt concerning the production of a true exotoxin by the Shiga bacillus and regard the toxic products as being entirely intracellular in location and origin. For example, Okell and Blake (57) made comparative observations on the viable count, toxin production, and *p*H of broth cultures of the Shiga bacillus during a prolonged period of incubation. Maximum multiplication of organisms was reached in about twelve hours, but a filtrate of the culture at this time was non-toxic for mice even in doses of 1 cc. Toxin was not demonstrable until after 24 hours and did not reach its maximum until after a number of days and after the *p*H had risen to 8.6 to 8.8. It was suggested by these observations that the toxin was formed within the cell and was not liberated until autolysis had taken place. Moreover, no toxin diffused out of dried bacillary cells when they were suspended in saline and shaken for several days. The dead bacillary bodies were very toxic. It should be pointed out that other organisms, notably the diphtheria bacillus, do not produce maximal exotoxin during the active growth phase.

The objections raised to the possible dual nature of Shiga bacillus toxin appear to have been answered by the recent studies of Boivin and Mesrobeanu (58, 59, 60, 61). These investigators have isolated the endotoxin in pure form and find it to be a complex gluco-lipid. It is not identifiable with the toxic substance (exotoxin) found in broth filtrates. Antitoxins produced by immunization with these separate toxins do not cross neutralize.

On the basis of our knowledge at the present time we must conclude that the Shiga bacillus produces a true, soluble exotoxin that is neutralized by antitoxin according to the law of multiple proportions. In addition, the bacillary cells contain a potent endotoxin that is not neutralized by anti-endotoxin according to this law.

The Shiga bacillus is the only organism in the dysentery group which produces a soluble exotoxin. All the others produce an endotoxin only of variable degrees of potency.

Prophylactic Vaccination against Dysentery. Numerous attempts have been made in the past to produce active immunity by the subcutaneous injection of suspensions of killed dysentery bacilli. Such a procedure proved impracticable because of the severe reactions following

the injection of even very small doses of vaccines. The reactions are probably due to the endotoxins of the organisms. Severe local reactions may persist for several days and these not uncommonly result in the formation of sterile abscesses.

Attempts have been made to reduce the toxicity of vaccines by treatment with various chemicals (62, 63). Olitsky (64), using the method of preparing lipo-vaccines introduced by LeMoignic and Pinoy (65, 66), recommended the suspension of heat-killed organisms in neutral almond oil. Less reaction follows the injection of such lipo-vaccines, probably because of the slow rate of absorption of the suspended material. The method has an important disadvantage in that it is difficult to be certain of the sterility of the product.

Shiga (67) employed a sero-vaccine consisting of the injection subcutaneously of a mixture of 0.5 cc. immune serum and 0.5 cc. of killed bacterial suspension. Three or four days later double the dose of vaccine was given without the addition of serum. Antibodies were demonstrable in the blood of vaccinated individuals 3 to 4 weeks later. Shiga inoculated a large number of individuals in Japan by this method and stated that the mortality was markedly reduced, although the morbidity rate was not appreciably altered. Gibson (68, 69) reported favorable results from the use of a sero-vaccine.

Dumas, Ramon, and Bilal (70) prepared a dysentery toxoid by formolinization of toxin. They reported the occurrence of mild reactions only following the subcutaneous injection of this material. The blood of vaccinated individuals, taken three weeks after a second injection of dysentery toxoid, contained enough antitoxin per cubic centimeter to neutralize from 5 to 20 M.L.D. of toxin for the rabbit.

Iguchi (71) and his associates employed a method of oral vaccination. They prepared pills containing 30 mg. of bacillary substance derived from three local strains. Three pills a day were given at three-day intervals. Figures are given reporting the results of this method of vaccination in a large number of infants, which indicate a reduction in morbidity but no appreciable reduction in mortality. Additional data are needed for confirmation.

In summary, it would appear that even a reasonably good case for prophylactic vaccination against bacillary dysentery has not been established. As we have already indicated, the reactions to a suitable vaccine preparation are such as to render their widespread use impractical. The large number of different serological types of organisms likewise limits their usefulness. It is of interest that the one homogeneous type, namely the Shiga bacillus, has gradually become a less frequent cause of dysentery, even in the Orient. We cannot, at the present time, recommend the use of dysentery vaccines as a general prophylactic measure.

The Therapeutic Use of Antisera in Bacillary Dysentery. Todd (72) and Rosenthal (73, 74) showed that the soluble toxin of the Shiga bacillus stimulated the production of neutralizing antitoxin which had protective properties on injection into animals. The serum prepared by Todd contained enough antitoxin per cubic centimeter to neutralize 20,000 M.L.D. for the rabbit. It was most effective when injected simultaneously with the toxin; its effectiveness diminished rapidly when administered even a short time after the injection of toxin or organisms.

Antiserum is prepared by the intravenous injection of horses with the soluble toxin of the Shiga bacillus or with the bacterial cells. Flexner and Amoss (75) prepared a polyvalent serum by the intravenous injection first of heat-killed and later living organisms (Shiga and Flexner bacilli). Undoubtedly the most desirable serum would be one containing a high titer of both anti-exotoxin and anti-endotoxin.

A method of standardizing Shiga bacillus antiserum has been described by Sudmersen, Runge, and O'Brien (76) and O'Brien and Runge (77). They prepared a test toxin by growing the organisms on nutrient agar for 48 hours, washing off the growth in distilled water, killing the organisms by heating to 58° to 60° C. for 10 minutes, centrifuging, and drying rapidly in a desiccator. The dried material was ground into a homogeneous powder.

A dose of 0.25 cc. of antitoxin C .950 was adopted as a standard test dose. This was the amount necessary to protect more than half the mice or rabbits injected with 0.2 mg. of their dried toxin which represented approximately ten lethal doses. Mice have gradually been adopted as the test animal, mixtures of toxin and antitoxin being injected intravenously. Blake and Okell (78) improved the method of standardization. They employed larger test doses of toxin. Zozaya (79) has described a polysaccharide precipitative test for the standardization of antibacterial dysentery serum. As Kurauchi and Nagata (80) have shown, this method is not suitable for estimating the antitoxin value of such sera.

It has been variously stated that the mortality from Shiga bacillus infections has been reduced by about one half by the use of antitoxin when it is administered in adequate dosage early in the course of the disease. The serum has usually been given either intramuscularly or intravenously. Reports have been contradictory concerning the value of antisera in other types of dysentery. In our opinion the use of serum should be restricted to the administration of antitoxin in early cases of proven Shiga bacillus infection. We do not recommend the use of either monovalent or polyvalent antisera in the treatment of cases due to other types of dysentery bacilli. The importance of accurate and rapid bacteriological diagnosis of the type of infection is, of course, obvious, in the management of patients with dysentery.

Asiatic Cholera

Prophylactic Vaccination in Asiatic Cholera. Attempts to protect human beings against cholera by prophylactic vaccination were made as early as 1885 by Ferran (81) in Spain. At the time when his experiments were done little was known regarding the production of immunity with killed cultures or with bacterial extracts, and under the influence of the French school and its endeavors to immunize with living attenuated organisms, he applied similar methods to cholera. First experimenting with guinea pigs, Ferran applied his method to human beings, inoculating them with small quantities of living broth cultures of cholera spirilla. In many of his experiments he gave, as the first injection, 8 drops of a fresh broth culture, following this, after 8 days, with 0.5 cc. of a similar culture. Severe local and general reactions followed these injections. The results were not striking, and Ferran's work was finally suppressed by the government.

Haffkine (82, 83, 84), a pupil of Pasteur's, likewise developed a method which depended upon the injection of living cultures, but he attempted, by an elaborate technique, to produce two separate vaccines, one attenuated, the other enhanced in virulence.

Attenuation was accomplished by growing the cholera vibrio at a temperature of 39° C. in broth over the surface of which a constant stream of sterile air was passed. Under these conditions the first crop of organisms died rapidly, but Haffkine practiced reinoculation into new broth flasks before complete death of the original culture had taken place; after a series of cultivations in this way he obtained cultures which produced merely temporary and slight edema when injected under the skin of guinea pigs. This weakened virus was used for the first inoculation.

He enhanced the virulence of cholera cultures by the following procedure:

a. Giving an animal an intraperitoneal injection of organisms larger than the fatal dose.

b. Removing the peritoneal exudate and exposing it for a few hours to the air.

c. Injecting this exudate into another animal and treating the resulting peritoneal exudate in the same way.

After a series of such animal passages he claimed to have obtained an organism of great virulence, and this was his second and stronger vaccine.

For applying the method to human beings Haffkine planted the organism upon agar slants, emulsified the growth in sterile water and injected 0.05 cc. to 0.2 cc. of such a suspension, using first the weak vaccine and five days later a more virulent culture. In many instances only a single injection was given. This method was used on large numbers of people on tea estates, in prisons, in regiments, etc., in India. It is difficult to appraise Haffkine's results because of numerous variables which occurred. The number of inoculated and uninoculated were often unequal. There

was frequently uneven risk of exposure and in some cases inoculation was practiced during the course of an epidemic. Greenwood and Yule (85) examined Haffkine's statistics and found that some of his results suggested some value for his vaccine. Haffkine (86) later abandoned his living vaccine and recommended the use of his second vaccine after killing the organisms by heating to 50° C. and adding 0.5 per cent phenol as a preservative.

Kolle (87), in 1896, introduced the use of a heat-killed vaccine preserved with 0.5 per cent phenol. He showed that such a vaccine, upon injection, stimulated the production of specific vibriocidal antibodies. His vaccine was used on a large scale by Murata (88) in Japan and Savas (89) in Greece. Some of the results of the latter investigator in particular seemed to indicate definite protective value (see Greenwood and Yule's statistical analysis).

According to Teague (90) and others, none of the more complicated modifications of cholera vaccine have any particular advantage over simple salt solution suspensions of young agar cultures killed by heating for one hour at 53° C. The vaccine made in the Government Laboratories in Bombay contains, according to Teague, eight thousand million organisms per cubic centimeter. It is administered in two doses of 0.5 and 1.0 cc. injected subcutaneously. This vaccine has been used extensively in India, and reports from there seem to indicate favorable results. According to Teague several million men in the Austrian and German armies were vaccinated during the World War. Thousands of them spent months in districts where the disease was present, and only a relatively small amount of cholera occurred among them.

At the present time anti-cholera vaccination is practiced extensively in various parts of the world. Heat-killed preparations are generally employed. While the procedure would appear to be of value, it is nevertheless difficult to appraise it with any degree of accuracy. The very nature of the epidemiology of the disease militates against an accurate assessment of the prophylactic value of vaccination.

Serum Therapy in Asiatic Cholera. Macfadyen (91), Schurupow (92), and others have prepared sera with fairly high anti-endotoxic titers. Such sera, in small amounts, protected animals against several lethal doses of endotoxin. The clinical results in man have not been particularly striking, and they are seldom employed at the present time.

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CHAPTER XXVII

WHOOPING COUGH

WHOOPING COUGH is an acute infectious disease of childhood characterized by an incubation period of 7 to 14 days, a catarrhal stage of about 7 days, followed by a period of paroxysmal coughing lasting from 2 to 6 weeks. The characteristic whoop is an inspiratory effort occurring at the end of a series of coughs. Vomiting of thick mucus frequently follows a paroxysm of coughing. At the height of the disease there is a marked leucocytosis with a considerable increase in the lymphocytes.

The mortality from whooping cough is particularly high during the first three years of life, especially during the first year. In the registration area of the United States the death rate from whooping cough is somewhat greater than that from measles and scarlet fever and is second among children's diseases only to diphtheria, while in Denmark and England it heads the list, having a higher death rate than measles, diphtheria, or scarlet fever. In the U. S. Public Health Service Report for 1928 it is stated that "in the registration area about 6000 children under 5 years died from whooping cough. This exceeds the deaths from diphtheria, scarlet fever, measles, or tuberculosis. It exceeds the deaths from measles and scarlet fever combined." Thus, it is only natural that so much effort should be expended to develop methods for prophylaxis and treatment.

Etiology. Before methods for specific prophylaxis can be developed in any infectious disease it is necessary that the etiology of that disease be established beyond doubt. Ever since *Hemophilus pertussis* was first described by Bordet and Gengou (1) as the causative agent of whooping cough, occasional reports have cast doubt on the etiological role of this organism. Rich (2) has reviewed this subject and has presented the following arguments to suggest that a filterable virus may be the etiological agent: (*a*) Pertussis bronchopneumonia is an interstitial reaction with mononuclear cells predominating, which is the characteristic histological response to infection with a virus. (*b*) Pertussis results in a permanent immunity as in most virus diseases, whereas most bacterial diseases do not. (Rich fails to mention that several virus diseases are characterized by a very short immunity, *i.e.*, herpes labialis, the common cold, influenza, etc., and that several bacterial

diseases — plague, typhoid fever, cholera — are followed by a lasting immunity.) (*c*) *Hemophilus pertussis* is isolated only during the early stages of the disease. (*d*) Experimental transmission of the disease (Rich, 1932) has not been adequately demonstrated and thus Koch's postulates are not fulfilled.

McCordock (3), Rich (2), and McCordock and Smith (4) have described intranuclear inclusion bodies in the lungs of children dying during the course of pertussis, which they feel suggests the possibility that a virus may be the primary etiological agent. It is suggested by Rich, however, that these inclusion bodies might be due to aspirated herpes virus. McCordock reviews a few instances from the literature where similar bodies were found in non-pertussis patients. Furthermore, the inclusion bodies described by Farber and Wolbach (5) in the salivary glands of a goodly percentage of infants dying from a variety of causes are morphologically indistinguishable from the inclusion bodies described by the above authors in the lungs of patients with pertussis.

Because of these suggestions that a virus may play a role in the etiology of pertussis, some recent transmission experiments have been done which, in our opinion, offer very conclusive proof that the Bordet-Gengou bacillus is the sole etiological agent in whooping cough. It should be pointed out that in the old transmission experiments reviewed by Rich in 1932, the dissociation of *Hemophilus pertussis* was not understood. It is likely that some of these experiments were done with "rough" avirulent strains.

In 1932 Rich and his associates (6) reported a series of important transmission experiments in chimpanzees. We quote directly their description of these experiments.

"Three apes, inoculated by spraying their throats with either Berkefeld V, W, or Seitz filtrates of sputum from early human cases of whooping cough and by subcutaneous inoculations of citrated blood from the same patients, developed febrile upper respiratory catarrhs after incubation periods of from five to seven days. A transfer of this catarrhal infection to a second ape was made by spraying its throat with a Berkefeld W filtrate of the rhinopharyngeal washings from an ape infected with human filtrate. Two apes inoculated with unfiltered pertussis sputum developed similar catarrhal affections after two- or three-day incubation periods. These catarrhs differed only in minor points, such as length of incubation period, and absence of leucocytosis from the typical picture of experimental common colds in the apes. No cough occurred during or following these affections with one exception, which will be noted later. Cultures of all filtrates remained sterile on appropriate media and Bordet bacilli were cultivated in each instance from the unfiltered sputa.

"One of two apes inoculated with unfiltered sputum developed a second catarrhal affection 30 days after being inoculated. Within a few days a typical paroxysmal cough appeared and was accompanied by a leukocytosis

and a marked absolute increase in the lymphocytes. Cough plates on Bordet medium on several occasions showed numerous Bordet bacilli. The cough lasted for seven weeks. Another ape was allowed to drink a heavy suspension of freshly isolated Bordet bacilli. After an incubation period of 24 days, this ape developed typical whooping cough. The cough was accompanied by a marked leukocytosis and an absolute increase in the lymphocytes. Cough plates were positive for Bordet bacilli. The cough endured for six weeks. A third ape, which was inoculated with third generation cultures of Bordet bacilli isolated from the previous ape, developed typical whooping cough after an incubation period of seven days. Again the cough was accompanied by a leukocytosis which at its height showed 63,000 white blood cells with an absolute increase in the lymphocytes to 44,730 cells. Cough plates were covered with Bordet bacilli. The cough persisted for six weeks. In all three apes, the complement fixation reaction with pertussis antigen, which was negative during the incubation period, became strongly positive after the disease was well developed. Berkefeld W. filtrates of the rhinopharyngeal washings taken during the catarrhal stage in two of these three apes and inoculated into other apes did not produce catarrhal affections."

These authors conclude that "the interpretation of these observations is difficult. There can be no doubt but that the three apes inoculated with pure cultures of Bordet bacilli or whole sputum developed typical whooping cough, which in its clinical and bacteriological aspects was indistinguishable from the human disease. However, the results in those apes inoculated with filtrates from human cases are inconclusive and are open to two interpretations. One, that the catarrhal affections represented the action of a hitherto unrecognized filterable virus which acts as the primary infecting agent in whooping cough, or that the catarrhal affections were simply common colds and that we were dealing with whooping cough cases who were also carriers of the virus of the common cold." In our opinion the latter explanation is the more likely.

In 1933 Sauer (7) inoculated (into the nose and throat) healthy young Rhesus and Ringtail monkeys with virulent Phase I cultures. In 8 of 76 instances the animals developed a paroxysmal cough followed by vomiting of mucus after an incubation period of 7 to 20 days. The organism recovered from one coughing animal produced the disease in another animal inoculated with it. These experiments are not conclusive, however, because of the small percentage of positive results.

Macdonald and Macdonald (8) reported a unique experiment on human volunteers. Four healthy boys whose medical histories were accurately known were used in the observations. Two of the boys had been vaccinated two months previously with the Sauer vaccine. The other two were non-immune. The throats of all four were sprayed with the filtrate of the first generation of a freshly isolated culture. No symptoms developed. The boys were kept rigidly quarantined in a

country camp. Eighteen days later a suspension containing 140 bacilli was dropped into the nose and throat of each boy. The non-immune boys developed typical whooping cough with positive cough plates. The vaccinated boys remained free of symptoms and had no positive cough plates even though they were all kept together throughout the period of observation. These authors concluded that the Bordet-Gengou bacillus is the sole cause of whooping cough and that active immunity is conferred by the injection of *Hemophilus pertussis* vaccine.

In Shibley's (9) transmission experiment the virus possibility was examined still further. This author carried a Phase I culture through 60 generations in order to be certain that a virus was not carried over in the culture. With this culture he succeeded in producing a typical train of symptoms in a chimpanzee. The animal was sacrificed and *Hemophilus pertussis* was recovered from various locations in the respiratory tract.

That a mononuclear interstitial bronchopneumonia can be caused by agents other than viruses was demonstrated by Sprunt, Martin, and Williams (10, 11). It was shown that various bacterial toxins (staphylococcus aureus, hemolytic streptococcus, and diphtheria) would cause similar changes when injected intratracheally. The same histological reaction was brought about by injection of pure cultures of either Bordet-Gengou or typhoid bacilli. They concluded that these pathological changes could not be differentiated from those occurring in children dying of pertussis or from those caused in animals by the viruses of epidemic influenza and psittacosis.

Because of the recent attempts at prophylactic vaccination, it is very pertinent to have a clear understanding of the etiology of the disease. Without reviewing the additional positive evidence in its favor, the evidence, in our opinion, is conclusive and overwhelming that the Bordet-Gengou bacillus is the sole cause of whooping cough. There is no pertinent evidence that a filterable virus plays a role.

Immunology of Whooping Cough. There is no immunity transmitted from mother to offspring as in measles, diphtheria, and scarlet fever. Newly born babies are completely susceptible to the disease. Indeed, it is during this age period that the mortality is so high. A single attack confers a lasting immunity. Diagnoses of second attacks are always to be regarded with suspicion, unless the diagnosis in each attack is confirmed by bacteriological examination. There are other conditions, such as influenzal bronchopneumonia and tracheobronchial lymphadenitis, which may cause a clinical picture similar to pertussis.

Complement-fixing and agglutinating antibodies have been described as occurring in the serum of patients with pertussis. These do not appear until late, however, and are of no diagnostic importance.

Kendrick, Gibbs, and Sprick (12) applied Huddleson's (13) opsonocytophagic test to a study of immunity to pertussis. Briefly, the test consists in observing the number of Phase I organisms phagocytized by the leucocytes in a specimen of citrated blood. The authors described their procedure in detail, together with a standardized system for recording results. During an attack of the disease there was a gradual increase in opsonin up to two months, following which it gradually declined. A positive result appeared too late in the disease to be of diagnostic importance. A similar rise occurred during the course of vaccination up to six months, followed by a gradual decline during the next one to two years. Singer-Brooks and Miller (14) have confirmed these findings and have pointed out a number of limitations and drawbacks to the test.

The immunological mechanism responsible for the permanence of immunity following an attack of whooping cough is not understood. It is reasonably certain that it is not dependent upon circulating, humoral antibodies. In experiments by Fothergill and Walker (15) it was impossible to demonstrate any difference in the bactericidal power of blood of non-immune and convalescent patients for virulent, Phase I pertussis bacilli. The blood of both non-immunes and immunes possessed considerable bactericidal power. This is of some interest, since positive blood cultures for this organism are never obtained. When the serum from the blood of these two groups was removed, inactivated at 56° C. for 30 minutes, and replaced, the bactericidal power was lost. Under these circumstances the blood from convalescents was not bactericidal.

It is possible that the immunity in pertussis is dependent entirely on cellular mechanisms. In fact, it has been suggested that it is due to a local tissue immunity in the respiratory tract. There is no direct evidence to support this theory, however.

Biology of Hemophilus Pertussis. Within the past few years there has been considerable advance in our knowledge of the biology and antigenic composition of the Bordet-Gengou bacillus which has an extremely important bearing on prophylaxis by vaccination. Leslie and Gardner (16) showed that *Hemophilus pertussis* is capable of undergoing dissociation into four serologically different variants, which they termed Phases I, II, III, and IV. This suggests a different antigenic composition in each of these phases. Lawson (17) and Shibley and Hoelscher (18) have confirmed the fact that this organism dissociates into an avirulent phase but were unable to observe the four distinct phases described by Leslie and Gardner. Toomey, Ranta, and Takacs (19) demonstrated a difference in the virulence for guinea pigs of "rough" and "smooth" cultures. Shibley and Hoelscher have shown a serological difference in these two forms. This serological difference between

the mutation forms probably accounts for the several types of pertussis bacilli described in the older literature. As far as we know now, *Hemophilus pertussis* is a homogeneous organism. Phase I is always hemolytic and is the form recovered in the disease. It possesses a capsule, a matter of considerable importance, as will be seen later. It is the virulent phase and corresponds to the "smooth" form in general dissociation nomenclature. The importance of this is that if any prophylactic vaccine is to be of value, it must be made from Phase I cultures.

Prophylaxis. Innumerable attempts have been made in the past to actively immunize children with *Hemophilus pertussis* vaccines. Smith (20) summarized most of the literature up to 1924 and was able to come to no conclusion regarding their value. Even Madsen's (21) unique experiences in the Faroe Islands were not entirely convincing. In light of our present knowledge there are several possible reasons for the failure of these vaccines. Many of them were probably made from dissociated, avirulent cultures. Immunization was generally not done a sufficiently long time before exposure (in fact, in most instances such vaccines were administered either after exposure or during the early stages of the disease). The method of preparation of the vaccine is of great importance.

Sauer (22, 23, 24, 25, 26) has reopened the entire question of whooping cough immunization. His experiences with his new type of vaccine, prepared in a manner to overcome some of the objections to the older preparations, have stimulated a renewed interest in the problem during the past few years.

Sauer's method of preparing and administering his vaccine is as follows (we quote excerpts from his papers directly):

"Bacillus pertussis vaccine (1 cc. = 10 billion bacilli) is made from recently isolated, strongly hemolytic (Phase I) strains, grown on Bordet (potato-glycerine extract agar) medium made with freshly defibrinated human blood. To minimize the culture medium content, the 48-hour growth is scraped off and mixed with 0.5 per cent phenolized physiologic solution of sodium chloride. To insure purity, a stained smear of each surface growth is examined before it is harvested. After a week in the refrigerator (during which time it is shaken daily), the concentrated suspension is cultured for sterility on three successive days. After dilution with 0.5 per cent phenolized physiological solution of sodium chloride so that 1 cc. contains about 10 billion bacilli, it is tubed, sealed and refrigerated until shortly before it is used."

Sauer has emphasized several points concerning the production of vaccine. It should be made with freshly isolated, Phase I cultures. It is made with human blood medium on the supposition that: (*a*) The organism is more apt to be maintained in its virulent phase on this medium. (*b*) Foreign blood protein from the medium cannot be taken

up with the vaccine, which eliminates the possibility of hypersensitive reactions.

A total of 8 cc. or 80 billion bacilli are given in three bilateral injections at weekly intervals. They are given subcutaneously in the deltoid and triceps regions. Subsequent injections should not be given in the same spot as the preceding in order to avoid the possibility of reactions. Care should also be exercised that the injections are not given intravenously. The first injection consists of 1 cc. in each arm, 1.5 cc. being given in each arm at the second and third injections.

Sauer's experiences with reactions following these injections are as follows:

"The local and systemic reactions are due to the vaccine (dead bacilli and endotoxin), not to the phenol or medium proteins. . . . Allergic reactions, sensitization to foreign protein or susceptibility to the Arthus phenomenon need not be feared, regardless of the amount of vaccine injected or the time interval between injections. . . . If a severe reaction follows an injection, the next injection may be postponed a few days, or only 1 cc. may be given (bilaterally) at subsequent injections and an extra one given a week after the third injection. The parents are forewarned of a transient rise in temperature, the temporary local reactions (redness, induration, and tenderness) and the subcutaneous nodules, which may persist for a few weeks at the site of each injection."

Since the first three years of life are the danger period in whooping cough mortality, prophylactic vaccination, if successful, should be done during infancy. Sauer recommends that it be done during the second half of the first year of life. It would also be advisable to immunize older non-immune children who have some chronic or debilitating disease such as tuberculosis, asthma, chronic pulmonary suppuration, etc.

One important point emphasized by Sauer is that whooping cough vaccine is of no value unless *it is given at least three months before exposure and is of no therapeutic value at any time during the course of the disease.* This, of course, is in keeping with the general immunological principles governing active immunization in any disease.

Results with Sauer's Vaccine. This investigator has published very convincing evidence as to the effectiveness of whooping cough vaccine. In one of his studies he was able to vaccinate certain children in individual families and leave the others as controls. During the course of five years 31 of such control children in 24 families developed whooping cough, whereas 29 vaccinated children in these same families who were intimately exposed during the incubation, catarrhal, and paroxysmal stages failed to develop the disease. During the same period 162 vaccinated children who were accidentally exposed failed to develop the disease. We have already quoted, in our discussion on etiology, the

experiences of Macdonald and Macdonald, which are excellent evidence for the effectiveness of prophylactic vaccination.

More recently Sauer has reported a few failures of his vaccine to protect. In a recent paper (25) he states that "about 10 per cent of the children injected with a total of 8 cc. of the approved commercial vaccine contracted pertussis when subsequently exposed to infection."

Kendrick and Eldering (27) published results of a well-controlled study of the effectiveness of *Hemophilus pertussis* vaccine for prophylaxis. Their series included 1592 children, 712 in the test group and 880 in the control group. In the total group there were 67 cases of whooping cough, of which 63 cases occurred in the control group. This would seem to be significant evidence that their vaccine, prepared with Phase I cultures and administered in relatively large doses, was effective in the prevention of whooping cough.

These authors (27, 28) point out that sheep blood is probably as effective as human blood as a medium constituent for cultures for vaccines. They report no sensitization reactions as a result of this. They further point out that as yet neither the optimum dosage of vaccine nor the time interval necessary for the development of immunity is known. We feel that these points are well taken. It is possible to maintain the Bordet-Gengou bacillus in its "smooth" phase for some time, regardless of the kind of blood used in the preparation of the medium.

Doull, Shibley, and McClelland (29) conducted a similar study in which they failed to confirm the results of Sauer and of Kendrick and Eldering. They harvested their organisms in distilled water, centrifuged and resuspended them in physiologic salt solution. Such a procedure undoubtedly results in the loss of important surface antigenic material. Miller (30) examined this possibility experimentally and showed that washing the organisms in either distilled water or salt solution partially removes specific substance which can be flocculated with immune serum. He states that "Apparently distilled water denatures the surface antigen in some way, whereas saline simply removes a part of it." In view of these findings washing should be omitted in the preparation of whooping cough vaccine. We cannot accept the experience of Doull and his co-workers as a failure to confirm the reported results of whooping cough immunization.

Krueger (31, 32, 33) and his associates have described a method for the production of a so-called pertussis undenatured endoantigen. Phase I organisms were grown on plates containing human blood. The cells were harvested in buffered, isotonic solution, thoroughly washed to remove metabolites, and then mechanically disrupted by grinding for twelve hours in a special type of ball mill. The resultant suspension was subjected to ultra-filtration through acetic collodion membranes

of such porosity that all intact cells were retained while material in solution or in a finely dispersed phase passed through. The *water clear* filtrate was stated to contain those constituents of the bacterial cell put into solution or suspension by physical rupture of the cell membranes. Each cubic centimeter contained the extract from 12 billion organisms.

We feel that there is insufficient evidence available to justify the assumption that this is an effective immunizing agent. Ultra-filtration may remove considerable immunizing substance. Furthermore, the method makes it difficult to quantitate do age in terms of the amount of original substance, although efforts have been made to standardize this extract on the basis of its nitrogen content. The main claim of these authors is that this antigen is undenatured because it has not been heated. We might point out again that the vaccines prepared by Sauer and by Kendrick and Eldering are not heated — in fact, they are subjected to much less physical manipulation than Krueger's endoantigen. Furthermore, Krueger's procedure may remove necessary capsular or surface antigenic material of a haptene nature. Such material should be preserved intact to result in the most effective immunizing agent. This has been clearly demonstrated in Miller's (30) experiments.

Frawley, Stallings, and Nichols (34) and Munns and Aldrich (35) have reported results on the use of Krueger's antigen *therapeutically* in which they claim definite benefit from it. We fail to see that they have proved this claim. We see no reason why Krueger's antigen should have any therapeutic value, at least in so far as such therapeutic value is dependent upon the development of an active immunity. Frawley (36) used the antigen prophylactically, and found that he did not get complete protection although he stated that the disease was milder. The fact that this antigen failed to protect when given before the onset of the disease is strong evidence, it seems to us, that it should be of no therapeutic value when given during the course of the disease.

Miller and Singer-Brooks (37) published a convincing study of the therapeutic value of Krueger's endoantigen in which they showed it to be of no value. The variability in the course of the natural disease may account for the evidence of therapeutic value described by some investigators in studies where the method of control was not adequate. The rigid control and the methods of collecting and appraising data make the results obtained by Miller and Singer-Brooks entirely convincing to us.

Duration of Immunity Following Vaccination. Sauer's studies are still too young to know definitely how long the immunity will last, granting it can be established by vaccination. It would seem from his studies that it will last longer than the period during which pertussis is such a dangerous disease. Sauer reported children who had been exposed from five to eight years after vaccination without developing

the disease. In view of the low index of infectiousness of pertussis, this is not absolute proof that vaccination immunity has lasted this long. It is logical to suppose that active immunity following vaccination will never be as durable or last as long as that following recovery from the disease.

Summary and Recommendations Regarding New Methods for Active Immunization against Pertussis. We feel that the evidence presented by Sauer, Kendrick and Eldering, and others is suggestive that a properly prepared and administered whooping cough vaccine has some prophylactic value. However, the last word concerning this cannot, as yet, be stated. Because of the low index of infectiousness, the uncertainty of a given exposure, etc., a great deal more adequately controlled statistical data will have to be gathered before one can make an exact statement as to the prophylactic value of the newer types of vaccine.

On the basis of the present evidence, however, we feel justified in recommending the use of a vaccine prepared from "whole," Phase I, non-heat-killed organisms which have not been washed, as a prophylactic agent only. In our opinion it is not necessary to prepare such vaccines by growing the organism on media containing human blood. Other bloods should serve as well. We do not recommend the use of specially prepared antigens or extracts of organisms. As we have pointed out elsewhere, the most effective vaccine is obtained by using virulent organisms of complete antigenic structure. This is obtained with most certainty by subjecting such organisms to the least possible physical or chemical manipulation.

Before using pertussis vaccine the physician should warn the parents of the child that no guarantee can be given as to its effectiveness. They should also be warned concerning the possibility of reactions.

Specific Therapy. Convalescent Serum and Vaccines. Numerous reports have been published on the use of convalescent serum or adult whole blood as either prophylactic or therapeutic agents. Bradford (38) has presented a review of the literature on this subject. In addition, he reported his own series of cases in which either convalescent serum (serum taken in the eighth week of convalescence) or adult whole blood was used. He concluded that these agents have some prophylactic value if given before the onset of symptoms of coryza and but little if any value as therapeutic agents after the disease has become established.

We feel that there is no good evidence that convalescent serum or whole blood possesses therapeutic value. The evidence concerning prophylactic value is likewise inconclusive. We have already pointed out that we were unable to demonstrate any difference in the bactericidal activity of convalescent blood as compared with blood from non-immune

patients. Even though such humoral antibody does exist and we had failed to demonstrate it, it should be pointed out that, in a sense, pertussis is largely a "surface type of infection." It is unlikely that parenterally injected antibody would reach the locus of infection in effective concentration because of the relative impermeability of the various interposing barriers.

Furthermore, convalescent serum provides passive immunity only, which, of course, is of very short duration. When we remember that Bordet-Gengou bacilli may be discharged from a patient for as long as three to five weeks, it is very unlikely that convalescent serum, given the other children in a family at the beginning of one child's illness, would protect throughout the entire period of this child's infectiousness.

We do not advise the use of convalescent serum or adult whole blood as either prophylactic or therapeutic agents in whooping cough.

For reasons which have already been given we feel that there is no justification for the use of any vaccines or bacterial extracts as therapeutic agents.

In conclusion, we should like to point out the great difficulty in properly controlling clinical observations designed to test the value of a specific or ameliorating agent in whooping cough, particularly therapeutic agents. The severity and duration of the disease are not only extremely variable but are *entirely unpredictable.* Therefore, no biological product can justifiably be said to have resulted in a "mild" disease in a particular individual patient, and any series of cases is necessarily composed of such individual patients. It is illogical, then, to classify groups of cases as "mild," "moderately severe," or "severe" with respect to the presumed effect of a therapeutic agent. It would not be too exacting to require that before a biological product can be accepted as having therapeutic value in whooping cough, it must cause a *prompt and complete cessation* of the disease with reasonable regularity.

The psychological effect of these therapeutic procedures in whooping cough is great, particularly on distraught parents and, in the last analysis, the value of a procedure, in most instances, is dependent upon their statements. For the child to receive "an injection for his whooping cough" often has a marked effect on parents.

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CHAPTER XXVIII

APPLIED IMMUNOLOGY IN SOME OTHER BACTERIAL INFECTIONS AND IN SNAKE-BITE POISONING

Plague

Prophylactic Immunization. The first attempts to immunize human beings against plague were those of Haffkine (1). The first vaccinations were carried out with broth cultures killed at 65° C. He tested his vaccines on a large scale in Bombay and obtained promising results. In a plague epidemic occurring in a Bombay prison only 2 of 151 vaccinated persons became ill, and neither of these died; whereas, of 177 unvaccinated persons 12 became ill and 6 died. In large series of vaccinated people only 1.8 per cent were infected with plague, with a mortality of 0.4 per cent for the total, whereas of unvaccinated individuals in the same epidemic 7.7 per cent fell victim to the disease, with a mortality of 4.7 per cent.

The German Plague Commission, consisting of Gaffky, Pfeiffer, and Dieudonné, recommended a vaccine of killed agar cultures. On the other hand, Kolle and Otto (2, 3), basing their results upon experiments carried out with monkeys, mice, guinea pigs, and rats, came to the conclusion that vaccination with dead plague cultures was much inferior to that obtained when attenuated living cultures were used. The same conclusion was reached by Kolle and Strong (4). Kolle and Otto found that immunization of animals with large doses of killed agar cultures of plague bacilli and with Haffkine's vaccine did not protect them against subsequent inoculation with virulent cultures.

Strong (5) subsequently made a comparative study of the various methods of plague vaccination, and concluded that the most efficient method was immunization with attenuated living cultures. He showed that when carefully done this method can be employed in human beings, but admitted that his work must still be considered as experimental and further studied before it could be universally employed. It is unlikely that any method so obviously attended by grave risks can ever be employed on a sufficiently large scale to make it practically significant.

Besredka (6) advised the use of sensitized dead plague cultures, claiming, from animal experimentation, that such vaccines produced an efficient and durable immunity. Rowland (7) confirmed the immu-

nizing properties of Besredka's vaccines and believed that the antigenic properties of the plague bacillus were attached to the bacterial nucleoproteins, and could be extracted with these. Rowland prepared a vaccine by the treatment of the moist bacteria with enough anhydrous sodium sulfate to combine with all the water present, froze and thawed the mixtures, then filtered off the bacterial deposits at 37° C., and extracted them with water. The solution so obtained was fatal to rats in small quantities and afforded substantial protection, reducing the mortality on subsequent inoculation of a standard culture from 80 to 10 per cent.

TABLE I

COMBINED SUMMARY OF FIGURES OF OUTBREAKS FOR WHICH COMPARABLE RELIABLE FIGURES ARE AVAILABLE

OUTBREAK	AVERAGE POPULATIONS		INOCULATED		UNINOCULATED	
	Inoculated	Uninoculated	Attacks	Deaths	Attacks	Deaths
House of correction, Byculla	147	172	2	—	12	6
Lanowli	323	377	14	7	78	57
Umerkhadi Jail	147	127	3	—	10	6
Undhera	71	64	8	3	27	26
Hubli	24,631	17,786	Not available	338	Not available	2,348
S. M. R., Hubli	1,260	760	11	2	35	21
Belgaum	4,842	4,558	78	40	506	346
Jewish Community, Aden	1,190	982	23	8	83	65
Nawashahr	2,648	2,141	9	5	41	32
Kahma	863	889	—	—	10	5
Khatkar Kalan	1,040	338	6	2	6	5
Salem	27,489	27,181	434	226	1,693	1,595
Coimbatore	26,559	22,604	150	56	707	492
Baghdad	56,555	108,445	65	36	806	638
Average population	147,765	186,424	—	—	—	—
Attacks and deaths	—	—	803*	723	4,014*	5,642

* Excluding Hubli.
Taken from Taylor (8).

Vaccination against plague has been used extensively in India. In a population of this sort it is notoriously difficult to collect reliable statistics; however, from figures recently compiled by Taylor (8) it would appear that both the attack rate and death rate have been significantly reduced. It is equally clear, also, that vaccination falls far short of assurance against an attack of the disease. The above table, taken from Taylor, summarizes the results from a series of inoculation cam-

paigns in which the data were sufficiently reliable to be subjected to statistical analysis.

Taylor (8) has presented the following calculations from this combined summary.

(*a*) Excluding Hubli:

	Inoculated	*Uninoculated*
Average population	123,134	168,638
Attacks	803	4,014
Deaths	385	3,294
Attack rate per 10,000	66	232
Death rate per 10,000	31	189
Case mortality	48 per cent	82 per cent

(*b*) Including Hubli:

	Inoculated	*Uninoculated*
Average population	147,765	186,424
Deaths	723	5,642
Death rate per 10,000	49	303

Plague vaccine is generally prepared by growing a highly virulent strain of the plague bacillus in a standard broth medium (an acid-digest of goat's flesh) for four weeks at 27° C. The vaccine is sterilized by heating at 55° C. for 15 minutes [see Taylor (8)]. Schütze (9) has reported that the alkali in the culture should be neutralized before heat sterilization in order not to destroy the surface or envelope antigen.

Various methods of standardizing plague vaccine have been proposed, but none are entirely satisfactory except perhaps the more recent efforts to develop a mouse-protective test by Taylor (8) and by Sokhey and Maurice (10). Bacterial counts are obviously unreliable because many organisms have died and autolyzed during the long period of incubation. The vaccine is generally prepared on the basis of a standardized procedure for the entire process. The adult dose is 4.0 cc. given in a single subcutaneous injection.

Schütze (11) has described two antigens for the plague bacillus, an envelope antigen which is heat labile and a heat-stable somatic antigen. Kurauchi and Homma (12) studied these two antigens, finding that the envelope antigen which they purified and concentrated by acid-acetone precipitation was the specific immunizing substance. They reported good results in active immunization experiments in animals with this substance.

The Serum Treatment of Plague. That the serum of animals immunized with killed plague cultures may protect normal animals from experimental infection was first shown by Yersin, Calmette, and Borrel (13). The serum which they produced possessed bactericidal action, but no antitoxic properties. They determined its protective powers by injecting measured quantities into mice and infecting them with fatal doses of virulent plague bacilli 24 hours later. The Yersin serum which was produced as a result of these experiments was made by the gradual

immunization of horses with, first, dead plague bacilli, finally with virulent living organisms. The serum was used with results that leave one much in doubt as to its efficacy. Yersin (14) himself, describing an epidemic in Nhatrang, reported a general mortality of 73 per cent for the whole epidemic, a mortality of 100 per cent in untreated cases, and of 42 per cent among those treated with his serum. However, these results were not accepted by all observers as proving the effectiveness of the serum, since the number of cases observed were few, and the irregularity in the gravity of the disease in different individuals makes statistical evidence unreliable unless large material can be studied.

Observations on the Yersin serum were published by the British Plague Commission (15). In this investigation the cases were controlled as to severity by blood culture, since it had been claimed by a number of earlier investigators that the Yersin serum was efficient in mild cases, but failed entirely in the severe ones. It appears from the report of this commission that ordinarily 70 per cent of cases of plague without bacilli in the blood survive, while three quarters of those with mild septicemia die, and all of those with a marked septicemia succumb. In the summary given of 146 cases treated with Yersin's serum by the British Commission 65.1 per cent died. These figures, together with an analysis of the percentages, classified according to the severity of the infections, do not show any significant curative action.

Markl (16, 17, 18), who claimed that the plague bacillus elaborated a soluble toxin, produced a plague serum by immunization of animals with filtrates of broth cultures. He claimed that 0.1 cc. of his serum protected animals against lethal doses of plague bacilli if given at the same time. He attributed much of its curative action to the fact that in the presence of this serum active phagocytosis takes place, which suggests to us that antibacterial antibody was present in his serum. Rowland prepared an immune serum in horses by inoculation with an extract of plague bacilli, his so-called "nucleoproteins," which appeared to have curative properties in rats.

These various sera were tried from time to time in the treatment of plague in man. The results were disappointing when used under well-controlled conditions such as in the studies of the Plague Commission.* In conclusion, it would appear that antiplague serum has no definite beneficial effect.

Tularemia

Tularemia is an acute infectious disease of rodents, which is frequently transmitted to man by insect bites or contact with infected animals, and

* For extensive literature on the general subject of plague the reader is referred to the various publications of the English Plague Commission appearing in *The Journal of Hygiene* between the years 1907 and 1915.

which has recently been observed in many parts of the world. In the United States wild rabbits constitute the main reservoir of infection, deer-flies and ticks being the important insect vectors. Cases have been reported from practically every state in the Union, but most of them from a few main foci.

The disease in man owes its peculiar character to the fact that while rodents have no resistance to the infection, man is moderately resistant; hence, while tularemia is a rapidly fatal septicemia in rabbits, it is usually a rather mild and often distressingly chronic disease in human beings. In this it resembles tuberculosis in some respects, and this similarity carries over from the clinical to the pathological and immunological aspects of the disease. The typical lesion is a miliary necrotic lesion, which heals with the appearance of epithelioid cells and fibroblasts at the periphery, often mistaken for a tubercle before the disease was widely known. As in tuberculosis, the type of disease produced seems to depend on a balance between the size of the infecting dose, the state of immunity of the individual, and the degree of allergy to the organism causing the disease.

Bacterium tularense is an organism so small that it is able to penetrate unbroken skin and to pass from generation to generation in the ova of infected ticks. The possible portals of entry are very numerous, but the most common are the skin of the hands or face, the mucous membranes of the nasopharynx and conjunctivae, and the intestinal tract. Usually a local lesion — an ulcer — appears at the portal of entry and with it a swelling of the lymph nodes draining this area [ulceroglandular and oculoglandular types (19)].

The incubation period is short, being from 1 to 12 days with 3 days the average, and the onset is generally characterized by chills, fever, and constitutional symptoms often preceding the actual appearance of the primary ulcer and glandular swelling. At this time, blood cultures are frequently positive and there occurs a dissemination of the organisms, producing miliary lesions in the viscera. In most cases the process localizes and runs its course with 2 to 3 weeks of fever. With an overwhelming infecting dose the sepsis may be maintained, producing the high sustained fever and prostration that characterize the so-called typhoid type of tularemia, which is at least 50 per cent fatal. According to Foshay (20) most fatal cases do not occur in this manner but arise from a later sepsis, due presumably to the ulceration of one of the first miliary lesions into a blood vessel, with dissemination of the organisms as in miliary tuberculosis. There is a good deal of pathological evidence for this view. Downs (21) has shown that similar events occur in experimental infection of partially immunized rabbits.

In general, tularemia is a mild disease. There has been a mortality of

4.8 per cent in 6174 cases occurring in the United States prior to 1936. It is not the mortality which makes the disease so important but the high morbidity among those whose occupation exposes them to risk of infection and the prolonged period of disability which the disease may produce. Although the febrile period usually lasts about three weeks, enlargement of the lymph nodes with suppuration and drainage may persist for months, and a few unfortunate patients have had chronic tularemia with relapses over a period of years. The organism is never easy to cultivate, guinea pig inoculation usually being necessary, and most positive cultures are obtained in the first few weeks. However, *Bact. tularense* may persist in the tissues for a long time, as is attested by its isolation from pleural fluid 3½ months (22) after onset and by pus from a bubo 5 months (23) after onset.

Immunologic Tests in Diagnosis. Since isolation of the etiological agent of tularemia is difficult by culture and dangerous by animal inoculation, immunological methods offer the most satisfactory means of proving a presumptive diagnosis. A number of such tests have been developed.

(*a*) Agglutination test with patient's serum. Agglutinins for *Bact. tularense* begin to appear during the second week of the disease, usually reaching a maximum titer 4 to 7 weeks after onset, and then gradually falling until titers of from 1 : 20 to 1 : 160 are common at the end of a year. Agglutinin titers frequently persist at this level or slightly lower for the rest of the patient's life, but disappear in a few cases. Maximum titers of 1 : 2560 or higher have been reported, but titers of 1 : 40 and higher are usually considered significant. High titers in experimental infections usually are associated with resistance, and at the time of blood stream dissemination in both man and partially immunized rabbits a fall in agglutinin titer occurs (21).

Cross agglutination with *Brucella melitensis* and *abortus* occurs in about 25 per cent of cases, and the converse seems true in Brucellosis (19). Usually the titers for *Bact. tularense* rise higher and more rapidly in tularemia, but a few cases have occurred where the agglutinins for both organisms were equally high throughout.

(*b*) Skin test with killed bacteria. The main objection to the agglutinin test is the fact that, like the Widal test, it does not become positive for 10 days or more. Foshay (24) has developed an intradermal test with killed organisms which gives a positive result earlier in the course of the disease. The antigen used is prepared from a heat-killed suspension by detoxification with nitrous acid as follows:

Two cubic centimeters of a suspension of heat-killed organisms, containing 10 billion bacilli per cc., are allowed to stand at room temperature for 24 hours after mixing with 2 cc. of fresh 30 per cent sodium nitrite solution and 2 cc. of 30 per cent acetic acid. The treated suspension is then washed several times with distilled water and saline and resuspended in 20 cc. of sterile saline giving a concentration of 1 billion organisms per cc.

For the test 0.01 cc. is injected intradermally. The positive reaction begins to appear in 36 hours with edema and erythema, which progresses

until at 48 hours there is a tender elevated area of erythema with a pale hard center. The reaction lasts several days and then slowly recedes, leaving a small area of brownish pigmentation.

This reaction appears to be very specific for infection with *Bact. tularense*, but like the agglutination reaction usually persists for years after the infection and, therefore, does not necessarily mean active disease at the time of the test. There has been no satisfactory report on cross reactions with Brucella antigens. None were obtained in a few patients tested, but in none of them was there any cross agglutination. The main advantage of this test lies in the ease with which it is performed and read and the fact that it becomes positive as early as the third or fourth day of the disease.

It is important to use the detoxified and diluted suspension, as heat-killed organisms or concentrated suspensions will produce severe necrotic skin reactions and marked constitutional disturbances in a highly sensitive patient. The reaction indicates hypersensitiveness of patients to the organism and is analogous to the tuberculin reaction in tuberculosis.

(*c*) Skin test with antiserum. Foshay (25) has also found that a patient who reacts positively to the bacterial skin test with a delayed reaction, as described above, gives an immediate reaction with erythema and wheal formation lasting 10 to 15 minutes to an intradermal injection of his antitularemia serum. This reaction is apparently specific; however, it must be controlled by giving another injection of normal serum from the same animal species, and should have a third control of immune serum from the same animal immunized against another organism. This reaction becomes positive early in the disease and persists long after recovery. It should not be confused with the reaction indicating hypersensitiveness to serum in preliminary testing before giving antiserum. It is difficult to understand the mechanism of production of this reaction. Additional analytical data are necessary to justify its acceptance as a new type of immunologic response.

In summary, the bacterial skin test is very valuable as an aid to early diagnosis, the agglutination test as a further check and means of following the patient's response to infection. Neither positive test necessarily means active infection, but if a negative skin test becomes positive or a negative or low titer agglutination reaction keeps increasing in intensity during the course of the disease, it is very strong evidence for active tularemia, provided Brucellosis can be ruled out.

Treatment with Antiserum. Although sporadic reports of successful treatment of tularemia patients with X ray, neoarsphenamine, mercurochrome, metaphen, and by other methods have appeared, the only therapeutic agent that has had anything like an adequate trial is Foshay's antiserum. In any disease so comparatively infrequent at any one place and time, so mild in general, and with such a chronic course, it is very difficult to form a sound judgment of any therapeutic program. Foshay has made a splendid attempt to keep accurate records of all cases treated, since he first developed his serum in 1931, and has published his results at intervals. The first reports in 1934 (26) and 1935 (27) were somewhat more favorable than the last, published in 1937 (20). The Council of

Pharmacy and Chemistry of the American Medical Association (28) decided that the results were too equivocal for acceptance of the commercial product as yet.

Foshay's statistics are given below. The general impression is that the course of the disease, the duration of disability, and the liability to suppurative adenitis are all measurably but not markedly reduced, particularly if the serum is given in the first 12 days of illness. Foshay stated that most patients feel subjectively improved within a day or two of treatment, even if the disease has persisted as a chronic case for several months. Treatment of the typhoid type has been unsatisfactory in everyone's hands, and Foshay stated that only two cases with obvious evidence of sepsis have recovered even with large doses of serum. However, in 1937, in writing of the cause of death in tularemia, Foshay stated that in 105 cases treated before the 12th day of illness and with no complicating disease, instead of the usual 5 or 6 fatalities, there was not a single death (20). Furthermore, no patient receiving serum before signs of sepsis appeared developed sepsis, and no patient receiving serum developed chronic tularemia.

TABLE II

COMPARATIVE DATA FROM TULAREMIA PATIENTS
(Means unless indicated otherwise)

	UNTREATED, 334 CASES	ALL TREATED, 481 CASES	TREATED 12TH DAY OR EARLIER, 133 CASES
Duration of disease (mos.)	4.10 ± 0.11	2.77 ± 0.05	2.32 ± 0.08
Disability (mos.)	3.11 ± 0.14	2.13 ± 0.04	1.62 ± 0.07
Adenopathies (mos.)	3.61 ± 0.08	2.32 ± 0.05	2.02 ± 0.08
Serum-recovery interval (days)	—	1.78 ± 0.04	1.99 ± 0.08
Fever (days)	27.92 ± 0.84	25.69 ± 0.53	20.19 ± 0.76
Primary lesions (days)	39.12 ± 2.14	31.59 ± 0.54	26.53 ± 0.84
Hospitalization (days)	30.51 ± 1.99	22.76 ± 0.55	21.17 ± 0.90
Suppurative adenitis, incidence	50.4%	42.0%	37.0%
Exanthem, incidence	No data	21.4%	—
Initial serum given, average day of disease	—	26.8	7.5
Mean (days)	—	26.34 ± 0.72	7.97 ± 0.18
Mode (days)	—	14.84	6.79
Mortality rate	5.8%	1.9%	3.0%

Taken from report of Dr. Foshay to Coun. on Pharm. and Chem., Am. Med. Assn. (28).

The recommended doses of serum are 30 cc. for an adult and 15 cc. for a child, given intravenously in divided doses on two consecutive days, but for a severe septic case much larger doses should be used.

The mode of action of the serum is not clear. It promotes specific

phagocytosis of the organism, and the agglutinins usually rise after its administration.

Active Immunization. The immunity conferred by one attack of tularemia, once it is over, is lasting, and no true second attacks have been reported. This is further emphasized by the persistence of agglutinins and positive skin tests for years after recovery, in one case for 22 years. Francis (29), who contracted typical tularemia in the laboratory when he first worked on the disease, has had four reinfections in the past 15 years. These have all consisted of small lesions at the sites of cuts on his fingers when heavily infected material was being handled. Two were accompanied by short-lived fever and slight glandular swelling, but at no time was he ill enough to stop work. Fully virulent organisms were cultured from these lesions despite the evanescent character of the infection.

There are no reports of active immunization in man. Francis and others have reported on the ineffectiveness of heat-killed organisms in producing immunity in rabbits. Downs succeeded in producing partial immunity in rabbits with injections of formalinized suspensions of organisms and suggested that such vaccines might be useful in man. Prophylactic immunization, if successful, would be very useful for laboratory workers, market men, hunters, shepherds in tick-infested regions, and others running a heavy risk of exposure.

Anthrax

Active Immunization against Anthrax. The method of active immunization, extensively used for the protection of cattle, sheep, and other domestic animals susceptible to anthrax, was first developed by Pasteur (30, 31). The principle of the method is the administration of anthrax bacilli which have been attenuated by cultivation at 42° C. to 43° C. in broth. The resultant strain, cultivated under these conditions for a sufficiently long time, is not only less virulent, but loses its power of spore formation. The reduction of virulence is in direct relationship to the length of time for which cultivation is continued. The relative virulence of a given culture can be estimated by a method described by Koch, Gaffky, and Loeffler (32). Rabbits are less susceptible than guinea pigs, and virulent anthrax cultures grown for two to three days under the conditions of increased temperature lose their power to kill rabbits in certain doses, but are still virulent for guinea pigs in these amounts. After 10 to 20 days of further cultivation at 42° C., the virulence for guinea pigs disappears, but the culture is still potent for mice. Even mouse virulence may eventually be eliminated by further cultivation at this temperature.

Pasteur's procedure was to grow anthrax cultures for different periods, under these conditions. A culture which had lost its virulence for guinea

pigs and rabbits and was still potent against mice was his "premier vaccin." A culture which was still definitely virulent for mice and guinea pigs, but no longer for rabbits, was his "deuxième vaccin." Such strains in which the attenuation had been definitely determined were grown in broth for 48 hours, and then injected into cattle in doses of 0.25 cc. and into sheep in about one half of this dose. The "premier vaccin" was injected first and 12 days later the "deuxième." The immunity was said to last one or even two years.

Numerous objections have been raised against this original Pasteurian method of vaccination. It is not possible to standardize accurately the virulence of these attenuated cultures. Pasteur thought it was possible to "fix" the virulence at a given point. Numerous studies such as those of Preisz (33) have shown this not to be true. A single culture may contain both virulent and avirulent variants of the organism. As a result other methods of prophylaxis have been developed which have largely replaced the technique of Pasteur.

Sobernheim (34, 35, 36) developed a method of combined active and passive immunization. He prepared immune sera in cattle and sheep by injections of cultures of increasing virulence. His vaccination procedure consists of the simultaneous subcutaneous injection of 10 cc. to 16 cc. of immune serum and 0.5 cc. to 1.0 cc. of a vaccine that has been slightly attenuated by growth at 42.5° C. His method is said to have given excellent results in Germany and South America, where it has been extensively used.

Eichhorn (37, 38) has developed a spore vaccine from tested strains in which he was able to maintain a standard virulence by preserving them in mixtures of glycerin and salt solution. The spore vaccine is injected simultaneously with immune serum. In districts where infection risk is great this procedure has been followed by injections of a No. 3 and No. 4 vaccine. Eichhorn, as well as others, has expressed the opinion that adequate immunity does not occur unless a definite reaction is produced by the vaccine in the animal receiving it.

Innumerable statistical reports have appeared since the development of anthrax vaccination by Pasteur which indicate that the procedure is of great value in the control of anthrax among domestic animals. The almost universal practice of vaccination would appear to attest to its value. On the other hand, numerous writers have pointed out that the evidence of benefit from vaccination is, actually, not as striking as would appear at first consideration. It is claimed that the incidence of the disease is still high in some countries, such as France, where vaccination has long been practiced. It is also pointed out that statistics showing a low incidence of disease among vaccinated animals are misleading because of a lack of control. The customary practice has

been to vaccinate all animals and compare the incidence of disease with that occurring during a previous period when vaccination was not practiced. Studies on a large scale where alternate animals have been vaccinated are conspicuously absent.

The Serum Treatment of Anthrax in Man. The greatest usefulness of anthrax antiserum has been in the treatment of the disease in man. Its greatest value has been in the treatment of cutaneous anthrax (malignant pustule). A number of sera have been developed and used for this purpose. Sclavo (39, 40, 41, 42) was among the first to develop a serum to be used extensively in man. Sclavo's serum as well as that of Sobernheim has been used extensively in Europe. Eichhorn's serum is most widely used in the United States. This serum has been concentrated by Eichhorn, Berg, and Kelser (43) by a method similar to that used for concentrating diphtheria antitoxin.

A numerous variety of methods of treatment have been employed. The more common of these in so far as serum is concerned have been the following:

(*a*) Administration of serum intravenously as well as locally in the area surrounding the lesion.

(*b*) Administration of serum intravenously only.

(*c*) A combination of serum administration by either or both of these routes, together with surgical excision or incision and drainage of the local lesion.

(*d*) A combination of serum administration by either or both of these routes, together with the injection of large doses of salvarsan.

It would appear that the first method yields the best results. It is generally agreed that it is unwise to employ surgical procedures in combination with serum therapy.

The dosage of serum has varied enormously in the hands of different observers. Some patients have been given huge doses. An average dose of serum for an adult would appear to be from 60 cc. to 80 cc. a day for the first two or three days, divided between the subcutaneous and intravenous routes.

From reports published by Sclavo (44), Legge (45), Hodgson (46), and Eurich (47) it would appear that the mortality from anthrax in man has been reduced from about 25 per cent to 5 to 8 per cent. A review of the literature on this subject will be found in the Report of the Committee on Industrial Anthrax of the American Public Health Association (48).

Gonococcus Infections

The use of specific immunologic procedures in the management of gonococcus infections is complicated at the outset by the fact that

N. gonorrheae is an antigenically complex group. Torrey (49) and Teague and Torrey (50) showed this by agglutination and agglutinin absorption, and Torrey separated these organisms into at least ten serological varieties. According to Watabiki (51) these strains shade into each other more or less in the same manner in which this is true of the meningococci. Hermanies (52) studied 85 strains and found that they fell into at least six distinct groups. From later studies by Torrey and Buckell (53), Tulloch (54, 55), and Atkin (56) it would appear that these organisms form at least two main types, with Type I being more commonly isolated from acute cases. There is, however, much overlapping of other strains between these types. Altogether, these organisms appear to be of a variable and complex antigenic composition with clear-cut classification being extremely difficult. Boor and Miller (57, 58) attempted a chemical fractionation of the antigens of the gonococcus and have described polysaccharide and nucleoprotein substances. They showed that these organisms have antigenic substances in common with other members of the Neisseriae and with the Type III pneumococcus.

Treatment of Gonorrhea with Vaccines. Although the literature on the beneficial effects of gonococcus vaccine is very confusing, there is no convincing evidence of the value of this procedure. Geraghty (59), in summarizing his results, stated that he believed there was no evidence indicating successful treatment of acute or subacute urethral infections. He stated, however, that he had seen occasional excellent results in acute complications such as arthritis and epididymitis. He added that in most instances in which he had seen favorable results, he observed acute reactions with temperature and leucocytosis, occasionally of sufficient severity to be alarming. He concluded, therefore, that since the vaccine was of absolutely no use in acute or chronic urethritis, and only occasionally gave symptomatic relief in the complications mentioned, the benefit derived was not proportionate to the risk incurred. We feel that the occasional benefit associated with reactions of this character may be due entirely to a foreign-protein-shock type of reaction.

Loeser (60), in 1929, reported on the use of a vaccine consisting of a suspension of living organisms prepared from young cultures of a freshly isolated strain. He used this vaccine in the treatment of chronic gonorrhea in women, giving it by subcutaneous injection. Some local reaction and a rise in temperature were considered essential to success. Others, including Herrold, Hoffman, and Blatt (61) and Felke (62), have reported success with this method. We fail to be convinced of the value of this method, believing that the injection of living organisms of this character into the body is a dangerous procedure.

From time to time various soluble products of the gonococcus have

been used in treatment either by injection or local application. The most recent of these which has received a measure of popularity in this country is the so-called "Corbus-Ferry filtrate" (63, 64, 65). A detailed and carefully controlled study of this product was published by Deakin (66), in which it was shown that this filtrate is of no value whatever in the treatment of gonorrhea. The Council on Pharmacy and Chemistry of the American Medical Association has ruled the Corbus-Ferry gonococcus filtrate not acceptable for inclusion in new and non-official remedies (67).

In conclusion, we fail to see that there is sufficient value in any gonococcal preparation of the nature of a vaccine or filtrate to justify their use. We do not recommend their use and feel more confident in this position in view of the good results being obtained by treatment with sulfanilamide.

Serum Treatment of Gonococcal Infections. A number of investigators have attempted the treatment of gonococcal infections with immune serum. In some instances these sera have been produced by immunization with whole organisms, in others by the injection of filtrates which undoubtedly contained various autolytic products of the organism. These sera have generally been employed in the treatment of certain complications, particularly gonococcal septicemia and arthritis. Reports on this form of therapy have been published by Torrey (68) and by Herrold (69) with some evidence of benefit.*

Spink and Keefer (70, 71) analyzed the mechanism of destruction of gonococci and found it to be a lysis of organisms due to the combined action of complement and antibody. Fresh, washed leucocytes had little or no killing action, even though the organisms were actively phagocyted. The addition of polyvalent antigonococcal serum to human blood greatly increased the killing power in *in vitro* experiments. Keefer and Spink (72) reported clinical results which indicated to them that it was justifiable to use antigonococcal serum in the treatment of patients with septicemia uncomplicated by endocarditis. They were unable, however, to sterilize the knee joint of a patient by injecting serum into it. In conclusion, it appears at the time of this writing that any benefit from serum is likely to be overshadowed by much more favorable results from the use of sulfanilamide.

Antivenin or Snake Poison Antitoxin

According to Calmette, Sewall was probably the first to experiment with the antigenic properties of snake poison and succeeded in increasing

* For a comprehensive review of the literature on the gonococcus and gonococcal infections the reader is referred to: Report of the Committee for Survey of Research on the Gonococcus and Gonococcal Infections, published as a supplement to *The Am. J. of Syphilis, Gonorrhea, and Venereal Diseases*, Vol. 20, No. 1. Jan., 1936.

the resistance of pigeons by systematic injections. Later Calmette (73, 74) and Phisalix and Bertrand (75) independently succeeded in producing immunity in rabbits and guinea pigs with cobra poison. Since then the poison of many different types of snakes has been found to be antigenic, and neutralizing antivenin against all but a few of them has been produced.

Investigations of snake poisons have had a very important influence upon immunological studies in general, since their heat stability made it possible for Calmette to show that toxin and antitoxin combinations are true neutralizations not accompanied by destruction of the toxin. These experiments were later elaborated by Morgenroth (76), who found that his hydrochloric acid combination with venom was so heat stable that, in neutral mixtures, it could be brought to the boiling point with complete destruction of the antitoxin and a liberation of the poisonous principle. The cobra poison seems to be more heat stable than that of the vipers, in which are included the rattlesnakes, copperheads, moccasins, etc.

There are many varieties of snake poison, the two chief classes being the poisons of the *Colubridae*, to which class the cobra belongs, and the *Viperidae*, which include the ordinary European vipers, the rattlesnakes, and most of the poisonous snakes of North and South America.

Snake poisons act both upon the blood, influencing coagulation, producing agglutination and hemolysis, and upon the nervous system. Descriptions of the effect of bites by cobras and by the African mamba characterize the very rapid effects as chiefly active upon the nervous system, secondarily affecting respiration and the heart. According to Calmette, 0.0002 gram of cobra poison will kill a guinea pig, and Noguchi states that 0.005 gram of rattlesnake venom will kill a guinea pig of 250 grams within twenty-four hours. There are certain snake poisons, also, which are characterized by specific action upon the endothelium of the blood vessels, producing edema and hemorrhages, effects studied by Flexner and Noguchi (77). The many studies of Do Amaral and others have shown that the venoms of different snakes contain many different elements. Moreover, it has been shown that the venoms of different types of snakes differ widely from each other in immunological aspects. The serum of horses hyperimmunized to rattlesnake venom gives relatively little protection against cobra poison or even against some of the closely related vipers. However, there is much immunological similarity between the poisons of the different species of cobra and between that of the genus *Bothrops* and the North American rattlesnake. Githens and Butz (78), for instance, have shown that the venoms of this species of North American rattlesnake contain almost identical poisons. The toxic principles of the copperhead and moccasin are

almost identical but differ somewhat from those of the rattlesnake. The poison of the *Crotalus terrificus*, the dog-faced rattlesnake of North and South America, and the *Bothrops atrox*, the fer-de-lance, contain principles differing entirely in immunological principle from rattlesnakes.*

It is therefore obvious that the production of a complete series of effective antivenins is a matter that cannot be accomplished until the knowledge of the antigens of different snakes has been thoroughly worked out. It is much more difficult to immunize against viper poisons than against the cobra poisons because of their more violent local reaction, which often causes necrosis. Nevertheless, it has been accomplished, and the gradual analysis of snake poisons is making it possible to supply antivenins that are highly potent against the more common snake poisons and somewhat potent against most others, except a few species against which it has not yet been possible to produce effective antivenins.

Directions for the management of snake bites have been given as follows by Do Amaral (79):

Immediate injection of antivenin, if available, or the following procedures should be immediately applied until antivenin can be procured.

Apply a ligature above the bite, tightly at first but partially released for a few seconds at five- or ten-minute intervals. There is no particular advantage in making an incision or in applying permanganate. "In fact," Do Amaral states, "it is advisable to avoid any further mutilation or injury of the affected tissue. In regard to potassium permanganate, it has been shown that in order to have any effect on the venom, it must be used in concentrations that are injurious to the tissues.

"Avoid the use of alcohol or any stimulant, since they tend to distribute the venom. Strychnine or caffein may be used for symptoms of weakness or giddiness.

"Proceed at once to the nearest place where anti-snake-bite serum is available. The North American snake venoms are slow, and if antivenin can be obtained within twelve to twenty-four hours the chances of its being effective are good."

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CHAPTER XXIX

APPLIED IMMUNOLOGY IN SOME VIRUS DISEASES

Measles (Morbilli)

MEASLES is a highly communicable acute exanthem characterized by a typical rash appearing first on the head and face and spreading over the trunk and limbs. The rash is preceded by a four-day period during which definite symptoms occur such as fever, coryza, conjunctival injection, and bronchitis. The diagnostic Koplik spots appear on the buccal mucous membrane during this prodromal period, while the skin rash is first evident at the height of this febrile period. Measles is one of the most striking of all diseases in that many of the more important features follow a very definite and predictable pattern.

While the general death rate from measles is low (about 0.2 per cent), there is, nevertheless, a large total number of deaths annually because of the great prevalence of the disease. The highest case fatality rate is during the first and second years of life.

Measles is to be regarded with much seriousness from the point of view of the secondary complications which may occur. The more frequent and serious of these are bronchopneumonia and pyogenic infections of the upper respiratory system, such as otitis media, mastoiditis, sinusitis, etc. Measles is very serious in children who have some other chronic or debilitating disease, particularly tuberculosis.

Etiology of Measles. The number of microorganisms that have, at one time or another, been proposed as the cause of measles is legion. Among these, the diplococcus of Tunnicliff (1) and of Ferry and Fisher (2) have received the most serious consideration, although convincing proof of their causal relation to the disease is entirely lacking. At the present time there is no described bacterium that can reasonably be assumed as the cause of measles.

The epidemiological and immunological evidence, together with no proven bacteriological evidence, has obliged most students of the problem to regard measles as being caused by an as yet undescribed, filterable virus. We ourselves subscribe to this opinion. It must be remembered, however, that such a virus has never been isolated so that its properties

could be studied, as in the case of many other filterable viruses. Recently, however, Torres and DeCastro (3) and Taniguchi and his associates (4) have claimed to have isolated and grown the causative agent in the chorio-allantoic membrane of chick embryos. These claims have not been confirmed. Rivers (5) has pointed out the ease with which experimental animals and the chorio-allantoic membrane of chick embryos may be contaminated with some other virus, especially if it is being worked with in the same laboratory at the same time.

The Experimental Transmission of Measles. Francis Home (6), in 1758, was apparently the first to attempt to produce measles in man by inoculation; his object being, apparently, to produce a milder form of the disease. It cannot be concluded from his description that measles resulted from these inoculations. Since then, numerous other workers have attempted to transmit measles to man but, as Hektoen (7) pointed out, the possibility of spontaneously occurring measles was not always sufficiently guarded against in most of the earlier experiments. Hektoen (8) apparently succeeded in transmitting measles to two human volunteers, although Sellards (9) was not successful in his attempts.

Innumerable attempts have been made to transmit measles to lower animals, including rabbits, guinea pigs, mice, cats, dogs, lambs, and pigs. While conflicting claims have been published, it cannot be said that measles has been transmitted to any of these animals. Hektoen (7) has thoroughly reviewed the literature on this subject.

Many attempts have been made to transmit measles to monkeys, and it seems likely that the disease has been produced in this animal. Goldberger and Anderson (10, 11, 12, 13) and Blake and Trask (14, 15) are generally credited with being successful, although Sellards and Wentworth (16) as well as others have recorded failures. The symptoms, which may include a shortened incubation period, a brief fever, leucopenia, respiratory symptoms, Koplik spots, and variable skin changes, are mild. On the basis of these transmission experiments, it would appear that the virus of measles is present in both the blood and nasopharyngeal secretions at least 24 hours before the rash and for a day or two after its appearance. The literature on the transmission and etiology of measles was reviewed by Hektoen (7) and Sellards (17).

Epidemiological Considerations. Many of the epidemiological facts about measles, such as the incubation period, etc., were clearly established by the remarkably accurate observations of Panum (18) in the Faroe Islands in 1846. The incubation period is ordinarily stated to be 14 days. This, however, is the period of time elapsing between the time of exposure and the first appearance of the rash. It is far more accurate to regard the incubation period as of 10 days' duration, or as the time elapsing between exposure and the appearance of the first

symptoms. The patient is suffering from the infection during this prodromal period. Indeed, this period is of greater epidemiological importance that any other, since the disease is more highly communicable during this time.

It is very desirable to have a precise definition of the infectious period during measles. For all practical purposes, this period can be considered from the first symptom (*i.e.*, 10 days after exposure) until the temperature has fallen (in the absence of secondary complications) and the rash has largely faded. Twelve to 14 days is an adequate isolation period. During the first 8 or 9 days of the incubation period, a child is of no danger to others. If the date and circumstances of the exposure are accurately known, there is nothing to be gained by restricting a child from school and other activities during this period.

The infectious agent of the disease is unquestionably spread from an infected to a susceptible individual by droplet infection. In this transfer, the virus probably leaves and enters the body by way of the respiratory passages. Thus, in spite of the extreme communicability of the disease, the infectious area surrounding a patient is quite limited. The extreme communicability is probably in large part due to the great susceptibility of the exposed individual, *i.e.*, a single and quite minimal infective dose of the virus is adequate.

There is every reason to suppose that the virus survives for an extremely short time outside the body. Its carriage on clothing, etc., by an intermediate person is restricted to a short period of time. There is no good evidence that carriers of measles virus exist in the sense of carriers of scarlet fever or diphtheria.

Immunological Considerations in Measles. The new-born infant is immune to measles if the mother has had the disease. This immunity lasts until the fourth or fifth month of life. It is a passive immunity, antibodies having reached the fetus through trans-placental transfer from the mother. From this fact alone it is evident that antibodies against measles are carried for many years in the blood of individuals recovered from the disease.

Susceptibility to measles is universal and complete. There is no gradual herd immunization without clinical evidence of the disease such as occurs in scarlet fever or diphtheria. No individual becomes immune without having the disease or a modified form of it as a result of partial protection by passive immunization. The claim is occasionally made by an adult individual that he or she has never had measles although many bona fide exposures have been experienced. Such claims can generally be attributed to a missed diagnosis in the individual's past. In this connection it should be realized that it is possible for an infant of four to six months of age, an age when the maternal passive immunity

may not have completely disappeared, to develop a modified and unrecognized attack of measles which might result in a permanent immunity. Of course, it is possible for an adequately isolated and protected individual never to develop measles during a lifetime. It must be insisted, nevertheless, that such would be extremely unlikely to occur if adequate exposure was experienced.

In addition to the completeness of susceptibility there is some evidence to indicate that measles is more virulent when an epidemic strikes an isolated population from which the disease has long been absent. This was suggested, in particular, by the devastating epidemics which occurred in the Sandwich Islands (18).

Immunity to measles is generally solid and permanent following an attack of the disease. Such careful observations as those of Chapin (18), however, suggest that second attacks do occur, although they are extremely rare.

Convalescent Serum, Adult Serum, and Adult Whole Blood in the Prevention and Modification of Measles. It would be beyond the practical scope of this discussion to review in detail the literature concerning the use of these materials in the prevention or modification of measles. Nicolle and Conseil (19, 20) are generally credited with being the first to use convalescent serum in the epidemiological control of the disease, although others had used it previously both as a preventive and therapeutic agent in individual cases. There is no doubt concerning the effectiveness of these reagents when used in adequate dosage and at the proper time in the incubation period.

Serum taken from an individual recently recovered from measles is very effective in preventing or modifying measles in an exposed individual. A dosage of 4 to 5 cc. of such serum given intramuscularly during the first four days after exposure will usually protect against measles. If modification is desired, the same dosage should be given later in the incubation period, between the fourth and eighth day after exposure.

A much larger dose of adult serum or serum of an individual who had measles a number of years previously is required; 15 cc. to 20 cc. should be given intramuscularly. Even with a much larger dose of adult serum the result is much less certain than with convalescent serum, as shown in the table on page 754.

Karelitz and Schick (22) and others have shown that the effectiveness of such sera is greater in institutions where isolation is practiced than it is in homes where the immunized child is repeatedly exposed to an infected brother or sister. These authors feel that the degree and frequency of infection are an important factor in determining whether measles will develop — a situation not unlike that in other infectious

TABLE I

A COMPARISON OF ADULT AND CONVALESCENT SERUM FOR THE PREVENTION OR MODIFICATION OF MEASLES

MATERIAL	NO. OF CASES	PROTECTED		MODIFIED		FAILED	
Adult serum . . .	584	329	56.4%	139	23.8%	116	19.8%
Convalescent serum	1627	1227	75.4%	273	16.8%	127	7.8%

Figures collected from the literature.
Taken from McKhann, C. F. (21).

diseases. It should be borne in mind, however, that there is a progressive diminution in the passive immunity throughout this period of exposure in the home.

The practice of using adult blood instead of adult serum or convalescent serum is rather popular for several reasons. Convalescent serum is not always available without the expenditure of considerable effort to get it. It is a simple, quick, and easy procedure to withdraw a syringeful of blood from an adult and inject it directly into the buttocks of a child. We definitely condemn this practice for several reasons. A large dose of adult blood, 30 cc. to 40 cc., is necessary to be effective. This is a painful, large mass to inject into the muscles of the buttocks of a small child. The hematoma thus produced may become secondarily infected and result in a severe sloughing and destructive wound. The infectious agent of some other disease such as syphilis or malaria may be unsuspectingly transferred from an adult to a child by such a procedure.

Collection and Distribution of Convalescent Serum. Various arrangements can and have been made for the collection, processing, preservation; and distribution of convalescent serum. Many of the larger contagious disease hospitals serve as depots. It is quite possible for local boards of health or other interested groups to arrange a depot for the collection and processing of convalescent serum.

There are certain precautions concerning the handling of convalescent serum and human placentas (for the production of placental extract) which must be met. Donors should be free from other communicable diseases such as syphilis. Sterility tests and other procedures to insure a safe product should be employed.

The "lyophile" process of Flosdorf and Mudd (23) is an effective method for concentrating convalescent or adult serum and preserving it for long periods of time with a minimum of deterioration. The pooled sera are preserved by drying *in vacuo* from the frozen state. The resulting dry, porous material ("lyophile" serum) may be redissolved in as

little as one quarter its original volume of liquid. Mudd and his associates (24, 25) have published reports concerning the use of human serum preserved by this technique in the prevention and treatment of various infectious diseases.

Placental Extract (McKhann). McKhann and his associates (26, 27, 28, 29) have prepared an extract from human placentae with which measles may be modified or prevented in much the same manner as with convalescent serum. Placental extract contains those immune bodies present in adult serum that are capable of passing the placental barrier to the fetus.

McKhann's method for preparing placental extract is briefly as follows:

"The organs are ground in a meat chopper and extracted with salt solution. After centrifugation of the saline extracts, which contain fetal blood serum, maternal blood serum and placental tissue protein, the globulins are precipitated by the addition of ammonium sulfate to one-half saturation. The precipitate is dialyzed to remove the ammonium sulfate, the product is filtered through paper pulp, passed through a supercentrifuge and then through Berkefeld filters. A preservative is added and after suitable tests for sterility and for reactions in animals and in humans, the material is issued for clinical use."

Results obtained by the use of placental extract are comparable to those obtained with convalescent and adult serum. Table II is a summary of McKhann's experience with this product.

TABLE II

PLACENTAL EXTRACT IN THE PREVENTION OR MODIFICATION OF MEASLES
INTRAMUSCULAR INJECTION

	DAY OF EXPOSURE	PROTECTION	MODIFICATION	FAILURE	TOTAL CASES
Intimate exposures given to protect	1–4	No. 282 56.1%	189 37.5%	32 6.4%	503
Given to modify . . .	5–12	No. 461 42.9%	531 49.4%	83 7.7%	1075
All exposures	1–12	No. 1740 65.3%	785 29.4%	140 5.3%	2665

The figures for intimate exposure include children exposed at home to a brother or a sister, or to a roommate in a boarding school, under which conditions more than 90 per cent of non-immune children may be expected to acquire the disease. In addition, any child exposed at school who developed measles has been transferred to the intimate exposure group. This increases the entire number of failures in this group, but it seems to be fair in that it shows they actually were exposed to the disease.

Taken from McKhann, C. F. (21).

The dosage of placental extract is difficult to state (varying between 5 cc. and 10 cc.) because of the absence of a practical direct method of standardization. The potency of each lot can be directly determined only by its actual use in the prevention or modification of measles. The material is administered by intramuscular injection.

Either local or general reactions have occurred with fair frequency following the injection of placental extract. In addition to these reactions McKhann has reported three instances of accelerated serum disease. This type of reaction presumably should not occur following the administration of human protein. It is possible, however, that the chemical manipulation during preparation may result in changes in the protein such that it is no longer homologous in a strict immunologic sense.

General Consideration of the Use of Convalescent Serum, Adult Serum, and Placental Extract in Measles. There can be no question but that these products have a useful place in the management of measles. They should be used to prevent measles under certain circumstances, such as in exposed children suffering from some chronic or debilitating illness such as tuberculosis, in very young infants, and to check an outbreak in a hospital ward or foundling home. These materials confer a passive immunity only. If complete prevention results, the immunity lasts for four to six weeks. Thus, an individual in whom complete prevention was accomplished is again susceptible to measles if exposed after this time. Another preventive dose will have to be given following such a subsequent exposure.

Whenever practical, such materials can be used in healthy exposed children in such a manner that modification of the disease can be expected. In modified measles the incubation period is slightly prolonged, the symptoms are milder (some of them may be absent), and secondary complications are uncommon. Modified measles results in active immunization which may be as solid as that following the natural disease. It must be remembered that a patient with modified measles is infectious and should be subjected to the same quarantine regulations as those for patients with the natural disease.

The use of these materials is not the final answer to the public health control of measles. The exposure must be known before they can be used. In order to have exposure there must be at least one case of measles among the particular group. Even in groups receiving adequate medical supervision unknown exposures will occur.

There is no evidence that these materials are of any value whatever in the treatment of measles even when given in large doses at the beginning of the prodromal symptoms. The occasional claims that the disease has been made milder by such treatment are not to be regarded as

indicating therapeutic value. That the disease may have been milder can logically be attributed to variation in its natural course. This conforms to the principles governing immunity in filterable virus diseases in general (see chapter on Immunity in Ultra-Microscopic Virus Diseases). In our opinion these materials should not be used for any purpose other than to attempt to prevent or modify the disease. *They are prophylactic agents only.*

Smallpox Vaccination

Prophylactic immunization against smallpox depends upon the principles discovered by Jenner in the eighteenth century. The original observation from which Jenner worked was that dairymaids and others who had been infected with cowpox were thereafter spared when a smallpox epidemic appeared in the region. It is now agreed by most observers who have studied the problem that the virus of cowpox and that of smallpox are identical, the former representing a strain attenuated by passage through cattle. This belief is based upon the observation that true variola can be transmitted to cattle, and that it can be carried from animal to animal, during this process becoming attenuated for human beings to such a degree that upon reinoculation into man a simple vaccinia is produced (30).

Smallpox prophylaxis, therefore, represents in principle active immunization with attenuated virus. When vaccination was first introduced, the virus was taken from pustules produced in other human beings. This has been given up today because of the dangers of transferring syphilis and other diseases in this way. At present the method of obtaining virus for vaccination is carried out as follows: The initial material consists of what is known as "seed" virus, which can be obtained from spontaneous cowpox or from vaccination pustules in children, or again from pustules obtained in calves after several passages of smallpox virus through calves. Calves may be inoculated from such seed virus or with material obtained from other calves in the usual way. At the present time the virulence of vaccine virus is generally maintained by intermittent passages through rabbits.

Healthy young animals are used; they are strapped down upon specially prepared tables, and the abdominal skin thoroughly cleansed with soap and water and shaved. The procedure varies in different places; often the skin is thoroughly cleansed with carbolic solution, and this is removed with sterile water before inoculation, or else cleansing is relied upon without the use of germicides. Over the clean area longitudinal scratches 1 to 2 cm. apart are made, and into these the seed virus is rubbed. The animals are then kept in a clean stall, preferably over asphalt floors, and rigid cleanliness observed. After the 6th or 7th day, when the vesicles are beginning to appear, the abdomen is thoroughly washed and cleansed of superficial dirt

without the use of an antiseptic and the pulp removed from the lesions with a curette. The pulp so removed is placed into 60 per cent glycerin and ground in a specially constructed mill. The animal should always be killed before the vesicles are removed, not only for humane reasons, but because a thorough autopsy can be performed to determine the health of the calf.

Vaccines so obtained always contain bacteria, the glycerin therefore serving a double purpose; one, the preservation of the virus, the other a gradual destruction of the bacteria. The addition of 2 to 4 parts of 60 per cent glycerin to one part by weight of the pulp prevents the growth of bacteria and probably destroys them by dehydration. Most of the bacteria are destroyed within one month. During this period, from 4 to 6 weeks, the glycerinated virus should not be used, and should from time to time be controlled by cultivation. At the end of this time the lymph is ready for use.

Formerly the material for the vaccination of human beings was obtained by dipping ivory splinters into the fluid of pustules, allowing this to dry, and rubbing these ivory or bone points into the exudate obtained by scratching the skin of the individual to be vaccinated. This method has gone out of use, and today the ripened glycerin pulp prepared as above is taken up in small capillary glass tubes and from these expressed upon the vaccination scratch. The potency of vaccine virus can be tested by the intradermal inoculation of rabbits with serial dilutions of the lymph.

That the ordinary vaccine virus ripened in glycerin is often ineffective in cases that might take if vaccinated with a more potent virus, such as the "green" or unripened vaccine, is a fact which has been forced upon our notice a number of times when soldiers of the United States Army, twice negatively vaccinated with glycerinated virus, developed takes after being vaccinated with unripened or "green" virus. This fact makes it very desirable that further research should be carried on in the production of more potent vaccines.

The first vaccination should preferably be done in infants at about 6 months of age. Infants may be vaccinated sooner than this in the event of an outbreak of the disease. Under ordinary circumstances a second vaccination should be done when an individual is 18 to 20 years old. Vaccination should always be repeated in the event of an exposure to the disease, an anticipated exposure, or an outbreak of the disease in the community.

The vaccination insertion is preferably made over the deltoid muscle of the upper arm. The area is cleansed with soap and water followed by some volatile antiseptic such as acetone. A drop of lymph is placed on the site. The superficial epidermis is pricked a number of times through the drop of lymph with a sterile steel needle held nearly parallel to the skin. The area of insertion need not be more than $\frac{1}{8}$ inch in diameter. An important factor in successful vaccination is to avoid

drawing blood. The excess lymph is gently wiped or absorbed off after waiting a few minutes after completing the insertion. The area need not be covered with a dressing and under no circumstances should a shield be used.

The practice of vaccinating young girls on the lower thigh is not recommended. Lesions in this location are more apt to become contaminated with dirt during the course of the child's play, thus creating an unnecessary risk of tetanus infection. (See chapter on Tetanus.) A negative vaccination should be followed in the same individual by a second attempt about two weeks later.

Vaccination if thoroughly and systematically done will unquestionably control smallpox without any additional measures. And failure to vaccinate will, as experience in many regions in the United States is demonstrating, invariably result in an increase in morbidity and mortality entirely inexcusable in a civilized country.

The disadvantages of current methods of vaccine production consist in the impossibility of obtaining completely sterile preparations except after prolonged storage in glycerine. During such storage there is inevitable attenuation of the vaccinia virus which leads to occasional failure of vaccination.

Efforts have therefore been made to produce a sterile vaccine material. In 1910 Henseval and Convent (31) found that if vaccinia virus was inoculated into the testicles of rabbits, it would there multiply and produce lesions in about four or five days. Noguchi (32) confirmed this and suggested that the testicular pulp be used for vaccination purposes. The method has not gained generalized application, partly because of the relatively small yield and partly because there is an opinion, not definitely established, that vaccinia virus may be attenuated for man by passage through rabbits.

A method which has not yet been tried out extensively on man but which has promise of possible advantages is that of producing potent vaccinia filtrates as described by Ward and Tang (33) and Ward (34). The great difficulties experienced by other workers in the filtration of vaccinia virus has been partly due to the intracellular position of the virus in the pulp and to the fact noted by the investigators mentioned, that filterable organisms pass through filters with great difficulty if suspended in salt solution, whereas suspension in broth makes it possible for them to pass easily. Moreover, it was found that, in order to filter pulp, the material must be fresh. For some unknown reason, storage even for one night in the ice-chest makes filtration much more difficult. By grinding fresh calf pulp with Pyrex glass dust and suspending in hormone broth, Ward and Tang succeeded in obtaining with great regularity a sterile filtrate of about $\frac{1}{16}$ the activity, volume for volume,

of a pulp emulsion used in human vaccination. Such virus could be partially concentrated by centrifugation and could be kept for long periods at or near the freezing point without deterioration.

Levaditi, Harvier, and Nicolau (35, 36) and Levaditi and Nicolau (37) succeeded in producing encephalitis in rabbits by intracerebral inoculation with vaccinia virus, and found that repeated passage through rabbits in this way increased the neurotropism of the virus until it reached a constant potency comparable to that of "virus fixé" in rabies. These investigators applied this bacteria-free neurovaccine to human vaccination. Of 12 vaccinations reported on new-born infants, 66 per cent were successful, and of 47 reported on nurslings (aged 17 days to 1 year) 71 per cent were successful. Secondary vesicles were encountered on three occasions and severe local reactions occurred twice. This "neurovaccine" has been used on many thousands of cases in Spain and France without, as far as we can learn, producing as large a percentage of postvaccinal encephalitis as has been observed in other countries using the ordinary vaccine. There is an innate theoretical risk, however, in the use of a virus specifically adapted to the nervous system. Moreover, the possibility of dissemination might be enhanced by growing the virus in any internal organ.

Production of Virus in Tissue and Chicken-Embryo Culture. With the advent of tissue culture and chick-embryo culture new fields have been opened up for the production of bacteria-free vaccinia virus. Extensive studies along these lines have been conducted by Rivers and Ward (38), Woodruff and Goodpasture (39), Buddingh (40), Stevenson and Butler (41), Godinho (42), Herzberg (43), and others.

The most elaborate study of the tissue-culture method was that carried on by Rivers and Ward (38). They carried a dermal strain of the virus through 99 successive tissue-culture passages (the medium consisting of 0.1 gram of viable chick-embryo tissue suspended in 4 to 5 cc. of Tyrode's solution). The rabbit titer slowly dropped from 1 : 1,000,000 to 1. Separate rejuvenated strains were made on the 34th and 88th passages by passing the virus through rabbit testes, which resulted in restoration of the original rabbit virulence. One hundred and eighteen individuals were inoculated with the culture virus (passages 5–88), both rejuvenated and un-rejuvenated strains, and in 100 a positive reaction occurred. The culture virus produced a refractory state to a standard dermal strain of calf lymph and *vice versa*. Several intradermal vaccinations were performed satisfactorily. These left little or no scar. Culture virus stored at 3° C. maintained activity for over a year. When desiccated and stored at 37° C., it exhibited some activity for five weeks.

Herzberg (43), in 1935, thoroughly studied the subject, using both egg chorio-allantoic membrane culture and Tyrode-egg embryo-tissue

culture. He inoculated 145 human cases and encountered no ill effects. No hemorrhagic or necrotic lesions were seen. The scratch method was used with material with a titer of 1 : 50,000.

Herzberg's Tyrode-tissue-cultures had a titer of 20,000 to 50,000 in 0.1 cc. (making it in amounts of 150 to 200 cc.). His egg embryo cultures titered 3 to 5 million. After storage in glycerin or on ice, the titer dropped in 3 months to $\frac{1}{3}$ to $\frac{1}{6}$ of the original value. He felt that egg embryo culture had some advantage because of the higher titer and because it could be stored in the crude form.

The use of egg membrane lymph dates back to 1931, when Woodruff and Goodpasture (39) reported a method of growing fowl pox virus in pure culture on the chorio-allantoic membrane of chick embryos. The method was then employed for growing vaccinia virus (44, 45), and the use of such virus for human vaccination was at once suggested. A thorough review of this work has been published by Buddingh (40).

The method used was to employ eggs containing live 14-day chick embryos. [Cf. Burnet (46) also.] After candling, a triangular flap of shell and shell membrane was raised. This exposed the chorio-allantoic membrane, which was inoculated with filtered calf lymph or material from previous eggs. The triangular flaps were then replaced and sealed with melted paraffin, following which the eggs were incubated at 37° C. for 72 hours. The membranes were harvested with aseptic technique and appropriate bacterial sterility tests made. Non-contaminated membranes were pooled, frozen, and ground up, and to each part was added four parts of 50 per cent glycerin in physiological salt solution or normal beef serum. Further bacteriological tests were done and the vaccine tested for potency on the rabbit.

When this material was stored at 5° C. or below, either in bulk or capillary tubes, no appreciable diminution of potency had occurred in one year and in the lower dilutions it produced good lesions after three years. The material compared favorably to calf lymph in potency.

Using this material it was found that the lesions produced in rabbits and man were slightly milder than with calf lymph. The evolution of the lesion was typical. Fever, malaise, local induration, and glandular involvement were less severe with chick vaccine and less scarring resulted. Immunity was about the same as with calf lymph. Revaccinations with calf lymph two years after vaccination with chick vaccine showed immune reactions in each case. Of 1120 vaccinations in both white and colored school children 93.6 per cent positive reactions were obtained. Inoculations were made with material obtained at various stages in the continuous passage of the virus.

Goodpasture and his co-workers have passed a dermal strain of virus through 220 successive generations during a period of five years. No alterations of its characteristics were noted, either in the lesions produced

on animals or man or in its titer for rabbits. Histological sections revealed that it produced the specific type of keratitis in the rabbits' cornea.

The glycerinated chick virus lost potency rapidly at temperatures higher than 5° C. It lost its viability completely in 10–12 days when stored at a temperature of 37° C. When sterile normal beef or rabbit serum was substituted for the 50 per cent glycerin, positive lesions were obtained after 6 weeks of storage at 37° C.

There is considerable disagreement as to change in virulence of vaccinia following repeated egg passage. Stevenson and Butler (41), Goodpasture and Buddingh (47), and Kunert (48) have found the virus to become less virulent with continuous egg passage. Lehmann (49), however, found the rabbit virulence to be much increased, with the lesions becoming more necrotic and hemorrhagic. This was also experienced by Tang and Wei (50), who inoculated a small number of human volunteers. One case developed a generalized eruption and fever.

All this would indicate the possible existence of several strains of vaccinia virus. We would agree with the suggestion of Buddingh (40) that it is necessary to use a dermal strain of virus which does not increase in virulence with the production of hemorrhagic, necrotic, and edematous lesions and which thus does not possess a potential danger of causing generalized vaccinia.

Certainly, enough evidence has been accumulated to demonstrate the practicability of the use of membrane lymph. Calf lymph, however, still has behind it the successful history of millions of effective vaccinations against smallpox and an established tradition for dependability that will be hard to uproot.

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CHAPTER XXX

APPLIED IMMUNOLOGY IN SOME OTHER VIRUS DISEASES

Acute Anterior Poliomyelitis

ANTERIOR POLIOMYELITIS is an acute virus disease involving the lower motor neurones of the central nervous system. It is a disease principally of childhood (1) and occurs in both epidemic and endemic form. There is a striking seasonal distribution (2, 3), although cases may occur at any time during the year. A unique and fortunate feature of the disease is that during any epidemic only a small proportion of the population is attacked. In this respect there is a close analogy to the incidence and distribution of cases during an epidemic of meningococcus meningitis.

The causative agent of this disease is one of the smaller of the filterable viruses, measuring from 12 to 17 mμ in diameter according to Theiler and Bauer (4). It is one of the least susceptible of all viruses to the influence of destructive agents. It is destroyed by heating to 50° C. for 30 minutes. Its activity persists for over a year in the presence of 0.5 per cent phenol, and it can be preserved for a number of years in 50 per cent glycerin. It was formerly thought that all strains were antigenically alike; however, during recent years evidence has accumulated suggesting the existence of more than one antigenic strain of the virus (5, 6, 7, 8).

Pathogenesis of Poliomyelitis. The infectious agent of this disease is unquestionably spread from individual to individual by direct contact. A number of theories have been advanced to explain the pathways by which the virus enters the tissues of the body and is transported to the cells in the spinal axis which it ultimately damages. At one time it was thought that poliomyelitis was a systemic disease at first with final localization in the anterior horns. It is generally agreed at present that the virus first enters the body through the olfactory area of the nose. In this area it enters the central nervous system directly by invading the olfactory hairs, which are freely exposed dendritic processes; thence it travels to the olfactory bulb. Its further progress from this site is through the central nervous system until it finally arrives in the anterior

horn area of the cerebrospinal axis. According to this theory the virus never invades any other tissue of the body except that of the central nervous system. There is a good deal of evidence to support this concept. In the first place, the virus has probably never been isolated from any tissue in man except the central nervous system and the nasopharynx. It has been demonstrated in filtered washings from this latter area. Furthermore, there has been a good deal of direct experimental evidence in monkeys to support this theory. This concept has an important bearing upon certain methods of prophylaxis that are being tried at the present time as well as upon a consideration of the value of specific therapy. Faber (9) has published a review of the literature supporting this theory of the pathogenesis of poliomyelitis.

From time to time another theory has been advocated, namely, that the virus first enters through the gastro-intestinal tract (10, 11, 12, 13). Toomey (14, 15, 16) has supported this theory. We agree with Flexner (17), however, that the evidence in support of it is not conclusive. We feel that the weight of evidence is in favor of the entrance of the virus through the mucous membrane of the olfactory area of the nose and of its direct passage through the cerebrospinal axis to the motor areas of the spinal cord and brain stem.

Natural Immunity to Poliomyelitis. It has long been recognized as an epidemiological fact that a single attack of the disease results in a durable and lasting immunity. Rare cases of bona fide secondary attacks have been reported, however. It has also been recognized that young infants fail to develop the disease. Furthermore, as we have pointed out above, epidemics of the disease are characterized by a very spotty distribution of cases and by the relatively small per cent of the population that is attacked even in a severe epidemic. An explanation for these features of the disease has been found in a study of the immunity of population samples as determined by the capacity of the serum of individuals to neutralize the activity of the virus. Such tests are performed by mixing appropriate quantities of serum and suspensions of spinal cord known to be active. Such mixtures are generally incubated for two hours, placed in an ice-box for 24 hours, and then injected intracerebrally into monkeys. Such tests are necessarily limited in their usefulness because of their expense.

Aycock and Kramer (18) have shown that the sera of a high percentage of urban adults are capable of neutralizing the virus. On the other hand, the sera of children between the ages of one and five years are usually lacking in this property. With advances in age an increasing percentage of individuals become immune to the disease as determined by this test. The curve describing the age-distribution of immunity is the reciprocal of that describing the age-distribution of the disease.

Aycock and Kramer have pointed out the close analogy between natural immunity in poliomyelitis and that in diphtheria. These investigators have also shown that the age distribution of the capacity to neutralize virus is somewhat different in rural than in urban areas. In rural areas this capacity develops much more slowly among the population and is probably due to the fact that such individuals, because of their isolated residence, have less contact with the virus.

Aycock and Kramer (19) performed neutralization tests on the cord blood of new-born infants as well as on the blood of their mothers. The blood of the infants was able to neutralize the virus in those instances where the maternal blood possessed a similar property. This was interpreted as being a passive immunity transferred through the placenta from the mother to her offspring, and being passively transferred, of relatively short duration. It probably accounts for the absence of the disease during infancy.

That a high percentage of an adult population is immune to the disease, even though very few of such individuals have had clinical evidence of it, is generally interpreted as indicating that during the seasons when the disease is common the virus must be exceedingly widespread. It suggests that many individuals have sufficient contact with the virus to produce immunity even though they never had manifestations of the disease. If we regard the virus as being distributed in this manner, the spotty distribution of cases, many of whom have no known contact with each other, is more easily understood. It would also appear that there are certain peculiar susceptibility factors involved to explain the development of the disease in a small percentage of individuals and not in others even though the latter must have had exposure to the virus as is evidenced by the later development of the capacity to neutralize it. During the past few years considerable evidence has been brought forward to indicate that many of the nondescript illnesses which occur during the summer and which are generally described by such terms as "summer complaints" may, in fact, be abortive cases of poliomyelitis (20, 21, 22). Such an occurrence would explain the development of immunity in some individuals without experiencing a typical attack of the disease. It also demonstrates the widespread distribution of the virus.

Active Immunization against Poliomyelitis. A great deal of work has been done in searching for some method of active immunization against this disease. Reference to our chapter on immunity in virus diseases in general will indicate the many problems attendant upon such an achievement, one of the most important of which is the great difficulty in immunizing with a so-called "killed" or inactive virus. We shall refer briefly at this time to a few of the most recent attempts in this direction

in regard to poliomyelitis. Aycock and Kagan (23) made a thorough study of this problem in 1927. They attempted to attenuate the virus by a variety of methods without success. Moreover, they investigated the possibility of immunization of monkeys by injecting active virus by various routes. They found that monkeys injected with large amounts of active virus subcutaneously or intradermally developed neutralizing substances in their serum; however, such animals were not always resistant to the intracerebral injection of virus. Stewart and Rhoads (24) published experiments indicating that monkeys could be immunized by the intracutaneous injection of active virus without developing the disease as a result of such injections. Obviously, of course, this procedure is too dangerous for use in man, particularly in view of the recent demonstration by Trask and Paul (6) of a strain of virus that is infectious for monkeys following its intradermal injection.

Rhoads (25) and Kramer (26) have immunized monkeys with neutral mixtures of virus and immune serum. Such a procedure was entirely safe, and some degree of immunity resulted; however, Kramer stated that "they [the immunized animals] could not be said to have possessed a solid immunity, comparable to animals who had recovered from a severe crippling attack of the disease."

Kolmer (27, 28, 29, 30) and his associates have treated poliomyelitis virus with sodium ricinoleate and claimed that such a procedure attenuated the virus without killing it. They reported numerous experiments in monkeys with their preparation and later employed the method for the immunization of children. It has since been shown by Kramer and Grossman (31) and by Olitsky and Cox (32) that this method does not, in fact, attenuate the virus. Leake (33), studying twelve cases occurring within three weeks after vaccination in areas free from epidemic, believes it impossible to exclude a possible relationship between the vaccinations and the attacks.

Brodie (34, 35, 36, 37, 38) has reported numerous experiments on the immunization of monkeys with a formalinized virus suspension. This method has been employed on a small scale for the vaccination of children. While it is likely that at least the major portion of the virus in such a preparation is inactivated, there is no conclusive evidence that it results in an effective immunization. Kramer (39) was unable to show that formalinized virus immunized experimental animals against infection following intracerebral inoculation of active material. He treated two series of monkeys. One was injected with 5.0 cc. of a 10 per cent cord suspension inactivated with formalin; the other with 10 cc. of the same suspension. The majority of these animals succumbed later to an intracerebral injection of an amount of active virus that was

slightly less than a constant minimal infective dose. Kramer also made observations on two groups of children. One group was injected with 5 cc. of formalinized virus. The serum of 50 per cent of these children contained neutralizing substances one to two months after vaccination. A similar group of children employed as controls were demonstrated not to have neutralizing substances in their sera at the beginning of the observations. When they were tested again, from one to three months later, 41 per cent had developed neutralizing substances in their sera. Kramer pointed out that there was no significant difference between these and the vaccinated group. Therefore, it cannot be assumed that vaccination was responsible for the development of neutralizing substances in the serum of the vaccinated group. Similar observations were made by Aycock and Hudson (40).

In conclusion, then, it is evident that no method has yet been developed for the safe and successful active immunization of man against poliomyelitis. We do not recommend the use of any of the procedures so far developed.

Prevention of Poliomyelitis by Chemical Methods. In 1934 Olitsky and Cox (41) observed that the daily intranasal instillation of a dilute solution of tannic acid on three successive days in mice protected these animals against the intranasal instillation of equine encephalomyelitis virus. The following year Armstrong and Harrison (42) reported that a similar instillation of a 4 per cent solution of sodium aluminum sulfate protected monkeys against intranasal infection by poliomyelitis virus. They believed that the protective effect was due to some alteration which decreased the permeability of the nasal mucous membrane rather than to an antiseptic action. Later, these authors (43) employed picric acid in a concentration of 0.32 to 0.64 per cent. They showed that this chemical was effective in preventing infection following the intranasal administration of encephalitis (St. Louis type) and poliomyelitis virus. The authors made 16 applications by means of an atomizer in their own nostrils and found that it caused no detectable injury. At about the same time Schultz and Gebhardt (44, 45) showed that the administration of immune serum failed to protect against intranasal infection with poliomyelitis virus. In a series of later communications Schultz and Gebhardt (46, 47, 48) reported numerous experiments in monkeys, as a result of which it appears conclusively demonstrated that properly applied chemical treatment to the olfactory area renders such animals insusceptible to intranasal infection by poliomyelitis virus for several weeks. These authors as well as Olitsky and Sabin (49) have made numerous studies of the comparative effectiveness of a large number of chemical agents. They are agreed that the most effective of these is a 1 per cent solution of zinc sulfate. They recommended the daily applica-

tion of this agent for three successive days to be followed by a single application at least once every two weeks during the time when risk of infection is great.

Peet, Echols, and Richter (50) made a detailed study of the application of this chemical in man. They found that when it was applied directly to the olfactory area there was a smarting sensation with some coryza, and for this reason they recommended that pontocaine hydrochloride be added to the preparation in 1 per cent solution. They found that it was much more difficult to spray the olfactory area in man than in monkeys. Applications with an atomizer and an ordinary tip were not satisfactory. They recommended that a special spray tip must be introduced to the region of the cribriform plate under direct vision and that at least 1 cc. of solution should be sprayed over each olfactory area. The services of an individual trained in the technique are required to carry out this procedure. When the olfactory area is properly and effectively sprayed, the sense of smell is temporarily lost but usually returns to normal in a week or two.

Tisdall (51) and his associates have reported on the use of this prophylactic method in a large group of children during a recent epidemic in Toronto. They organized an elaborate system of clinics for giving such treatments, all of which were done by experienced otolaryngologists. The recommendations of Peet, Echols, and Richter were followed except that it was found impractical to administer a daily spray for three successive days. Two sprays at an interval of two weeks were administered. It was found that the sense of smell was lost in slightly less than 25 per cent of a large sample that was tested. They included in their study a treated group of 4713 children and a representative control group of 6300 children. The first spraying was commenced on August 31 and completed September 5, the second spraying being carried out two weeks later. Eleven cases of poliomyelitis occurred in the sprayed group between this time and the conclusion of observations late in October, while 18 cases occurred during the same period among the larger group of control children. These investigators analyzed their figures in various ways and showed that there was no statistically significant difference in the attack rates in the two groups. They concluded that spraying the olfactory area of man with zinc sulfate was of no protective value. They also concluded that the difficulty of application, the equipment needed, the trained personnel, etc., rendered the procedure not suitable as a general public health measure in the face of an emergency outbreak. While this report of Tisdall and his co-workers is definitely discouraging, nevertheless, we will have to await additional large-scale studies in man before we are justified in forming a definite opinion as to the value of the procedure.

The Treatment of Poliomyelitis with Convalescent Serum. Netter (52) was apparently the first to use convalescent serum in the treatment of poliomyelitis; however, his cases were paralyzed when treatment was started. Later Amoss and Chesney (53), Draper (54), Peabody (55), Schwarz (56), and others employed this method during the preparalytic stage of the disease.

In 1928 Aycock and Luther (57) reported on an extensive study of the use of convalescent serum in 106 cases treated before the onset of paralysis. They developed a detailed method of muscle examination by which the amount of paralysis could be expressed numerically. Excluding one fatal case, 64 per cent of the remainder subsequently developed paralysis. The total amount of paralysis in the treated group was 19 as compared with 63.6 in 482 untreated cases. It was obvious that this form of therapy did not prevent paralysis. These authors felt, however, that it reduced the mortality and was responsible for a lowered average total paralysis. These results were confirmed and extended in a subsequent report by Aycock (58) and his associates.

Aycock, Luther, and Kramer (59) presented a detailed description of their method of collecting, processing, testing, and administering convalescent serum. Their treatment routine was as follows: At the time of the diagnostic lumbar puncture, 20 cc. of serum was injected intrathecally, if more than this amount of cerebrospinal fluid was obtained and if the cell count was characteristic. The patient was then given an intravenous injection of 60 cc. of serum. On the following day an additional 20 cc. was injected intrathecally.

Reactions occurred frequently following the intraspinous injection of convalescent serum and generally consisted of an exaggeration of meningeal signs such as an increased stiffness of the neck and back and the presence of Kernig's sign. There was often a marked increase in the cell count of the cerebrospinal fluid. Chills and hyperthermic reactions occurred occasionally.

In view of our present concept of the pathogenesis of poliomyelitis there would appear to be no rationale for the intraspinous administration of serum. It is likely that immune bodies are brought to the site of involvement by the blood stream only, if at all. Furthermore, the irritative reaction in the meninges may be harmful.

During the past few years numerous additional reports have appeared on the therapeutic use of convalescent serum with an increase in the expressions of doubt as to the value of the method. As improvement in early diagnosis has occurred it has become evident that many cases of preparalytic poliomyelitis do not go on to paralysis. In a later report by Kramer *et al.* (60), in which approximately alternate cases were treated, it was concluded that no statistically significant evidence of

benefit from convalescent serum was obtained. In 1932 Park (61) reported the results of a large experience with this treatment in New York City. His observations were made on 519 treated cases and 408 controls. No difference was observed in the results in the treated and untreated groups. Similar results were reported by Fischer (62).

In our opinion there is no evidence, either clinical or experimental, that convalescent serum is of any benefit in the treatment of acute poliomyelitis. (See chapter on immunity in virus diseases in general.)

Rabies

The most frequent problem with which the practicing physician is confronted in connection with rabies is the decision as to whether treatment should be instituted in the case of an individual bitten by a dog or cat. In all such cases the first thing to make sure of is that the animal in question is not killed, but held under observation, preferably by a veterinarian. If the animal shows no symptoms whatever for a week after the bite, danger of rabies may be excluded. As far as we know, the saliva of a rabid animal does not become infectious until within a week before the appearance of symptoms.

Characteristics of rabies in a dog are first, restlessness, unwillingness to play, loss of appetite, photophobia, and consequent crawling away in a dark corner. After that, the jaw may drop, the facial expression change, the bark become high pitched, and salivation develops as a consequence of difficulty in swallowing. This is often followed within a day or two by motor irritation, running, yelping, and excitability, which lead gradually into a convulsive stage in which the animal will die. The brain of the animal should always be sent to a laboratory for Negri body examination and animal inoculation.

If characteristic symptoms develop within a week after the bite and Negri bodies are found, the bitten individual should be treated. Any other individuals who have come into infectious contact with saliva should be considered for treatment. The decision in such cases is often very difficult, and no doubt many people are needlessly treated. But rabies once developed is fatal and in cases of reasonable doubt it is always safer to treat.

Choice of Method. The principle of treatment in rabies is an effort to immunize actively during the period of incubation, which fortunately is long — averaging about 40 to 60 days. It is supposed to be shortest in cases where bites are around the head and neck, since it is believed that the virus moves centripetally along the peripheral nerves.

The idea underlying Pasteur's (63) * original method of treatment

* Various papers reporting the experimental work of Pasteur and his associates on rabies will be found in the Compt. rend. Acad. sc., from the years 1881 to 1886.

was that the ordinary "street" virus of rabid animals could be raised to a constancy of virulence by passage through rabbits, and that such "virus fixé," which consists of the central nervous system (the cords) of animals that had died of this passage material, could then be systematically attenuated by drying over sodium hydroxide at about 22° C. Subdurally inoculated in rabbits, "virus fixé" so dried for 5 days produces rabies in about 8 days; dried for 9 days it kills in about 15 days, and dried for 14 days it becomes avirulent. The original method of immunization then consisted in starting with the injection of material that had been dried for as long as two weeks, and, in successive daily injections, administering "virus fixé" material that had been less and less completely attenuated. In the course of subsequent experience, it was no longer found necessary to begin with the 14-day cord. The following 21-day scheme of rabies vaccination is illustrative of the method used.

TWENTY-ONE DAY SCHEME OF RABIES VACCINE TREATMENT

DAY	DAYS CORD DRIED	FACE CASES	NUMBER OF INJECTIONS	ADULTS	CHILDREN	
					6 to 10 years	1 to 5 years
1	8—7—6	8—7—6	2	3 cc.	3 cc.	3 cc.
2	8—7—6	8—7—6	2	3 cc.	3 cc.	3 cc.
3	5—4	5—4	2	3 cc.	3 cc.	3 cc.
4	5	5	1	2 cc.	2 cc.	2 cc.
5	4	4	1	2 cc.	2 cc.	* 1½ cc.
6	4	4	1	2 cc.	2 cc.	* 1½ cc.
7	3	3	1	2 cc.	* 1½ cc.	* 1 cc.
8	3	3	1	2 cc.	* 1½ cc.	2 cc.
9	5	2	1	2 cc.	2 cc.	2 cc.
10	4	4	1	2 cc.	2 cc.	2 cc.
11	4	4	1	2 cc.	2 cc.	2 cc.
12	3	3	1	2 cc.	2 cc.	* 1½ cc.
13	3	2	1	2 cc.	2 cc.	* 1½ cc.
14	4	4	1	2 cc.	2 cc.	2 cc.
15	4	4	1	2 cc.	2 cc.	2 cc.
16	3	3	1	2 cc.	2 cc.	* 1½ cc.
17	3	2	1	2 cc.	2 cc.	* 1½ cc.
18	4	4	1	2 cc.	2 cc.	2 cc.
19	4	4	1	2 cc.	2 cc.	2 cc.
20	3	3	1	2 cc.	2 cc.	2 cc.
21	3	2	1	2 cc.	2 cc.	2 cc.

* Face cases receive adult doses.

Many other systems of rabies immunization based on the same principles have been devised. The method of Högyes (64) consists of diluting "virus fixé" and starting injections with high dilutions, gradually increasing the amounts administered. The method of Harris (65, 66) consisted in freezing "virus fixé" with carbon dioxide snow,

grinding it up, drying *in vacuo*, and preserving at temperatures of − 18° to − 20° C. Neither of these methods is in extensive use at the present time.

Another method which is displacing the original Pasteur procedure is that of Semple (67, 68). "Virus fixé" is ground up in salt solution in a concentration of about 8 per cent, exposed to 1 per cent carbolic acid at 37° C. for about 24 hours. After this time the fluid is diluted with an equal amount of normal salt solution, making a 4 per cent rabies virus in 0.5 per cent carbolic acid. This material is used for the so-called 15-injection treatment, daily injections of about 2.5 cc. being given for 15 days. The Semple method has been approved and licensed by the United States Government and most of the state governments.

The only other method of attenuating rabies virus for immunization purposes which has attained any degree of prominence is that of Kelser (69), who made a thin paste of "virus fixé" by grinding the material in the proportion of about 1 gram to 2 cc. of salt solution. The mixture was passed through an 80-mesh wire sieve and placed in a bottle with 1 per cent c.p. chloroform. This material was kept in the refrigerator and allowed to stand with daily shaking for a period of two weeks before use. The chloroform virus has been used chiefly in veterinary practice.

For appraisal of the effectiveness of anti-rabies vaccination we are to a large extent dependent upon statistical evidence, and in this case statistics are particularly difficult to evaluate for the following reasons: In countries where reliable statistics are available, practically all cases of bites from rabies-positive dogs are treated and there are consequently no untreated controls. There are naturally a great many cases of treatment in which there is no proof of rabies in the dog, and such cases are included in most statistics. The possible instance of rabies in individuals bitten by rabid dogs is necessarily variable, dependent upon the stage of rabies in the animal at the time of the bite, the interposition of clothing, and the indeterminable factors of uncertainty which are apparent in the irregularity with which rabies can be produced experimentally by subcutaneous injections, even of large quantities of street virus into susceptible animals. We are, therefore, not in a position to make categorical statements about the effectiveness of any kind of treatment. However, the enormous accumulation of data over prolonged periods during which hundreds of thousands of people have been treated by the Pasteur and other methods with not more than 1 per cent mortality among the treated, forms a strong argument in favor of effectiveness.

Cornwall's (70) statistics in India, though, indeed, based on a relatively small number of persons, indicate the possibility of a mortality up to 35 per cent among untreated persons bitten by dogs found positive.

And in another series in which the same writer estimates the effect of treatment on 28,898 individuals he finds a mortality among the treated of only 0.1 per cent. In this last series, however, the cases treated included many in whom the bites had been inflicted by animals not examined for Negri bodies. In a further study, the same writer gathered data upon cases in which a presumably rabid dog had bitten several people, some of whom accepted treatment and some of whom refused (a total of 2174 cases). He found that the death rate among the treated was 2.9 per cent as against 6.2 per cent among the untreated.

The puzzling problem in rabies immunization is the one concerning the immunizing power of dead virus. In injecting either the Pasteur material or the Semple or the chloroform virus, are we injecting killed virus or is our treatment effective only if a certain amount of living material is left in the suspensions? It has been impossible to solve this question with certainty because there was hitherto no available method of producing experimental rabies in animals with constancy except by direct subdural intracerebral or intraocular injection, all of them methods quite different from the mode of infection occurring when the human being is bitten by a rabid animal. Indeed, the injection of large amounts of street virus or "virus fixé" subcutaneously into rabbits and guinea pigs very often produces no disease whatever. It is not possible, therefore, to test out the immunizing power of any of the preparations through the treatment of animals and their subsequent infection by methods analogous to the accidental inoculation of a bite. Experience of the last few years has indeed revealed individual cases in which prophylactic rabies treatment properly carried out and started in time did not protect, and a few cases have been observed in dogs in which supposedly chloroform-killed virus has still been infectious. Fortunately, the recent results obtained by Webster (71, 72, 73, 74) and his associates with mice give promise of eventual solution of this important question. Webster worked with a special strain of Swiss mice which are uniformly and highly susceptible to rabies virus. Such mice may succumb to intracerebral injection of 10^{-5} dilution of rabies virus and often 10^{-1} dilution injected intraperitoneally. Given such a test animal he was able then to develop a protection test by which he can determine to what extent the injection of prophylactic preparations will produce viricidal bodies in the sera of treated individuals. In so testing a considerable number of commercial and Board of Health preparations of Semple vaccine he found that only a few developed effective viricidal bodies against 10 to 100 mouse intracerebral lethal doses. This method has not yet been fully exploited but undoubtedly offers the opportunity for the eventual determination of the effectiveness of rabies preparations.

It is not possible as yet, therefore, to say whether in the approved

methods of immunization we are treating with large amounts of killed virus, with highly attenuated virus, or with residues of virulent living material. It is reasonably certain, however, that the two chief methods employed are to some extent effective, and the physician has no choice but to proceed with a course of injections of the Pasteur or preferably the Semple preparations as soon as the rabic condition of the animal has been established.

The successful cultivation of rabies virus in Maitland tissue cultures within the last few years will unquestionably lead to clearer understanding of the entire situation and to changes in our methods of preparing vaccine (75, 76).

Treatment Paralyses. Occasionally, though rarely, in the course of antirabies treatment certain neurological accidents occur which have given rise to much discussion. These occurrences are extremely rare. Remlinger (77), who studied their frequency for the League of Nations Report of 1927, was able to find only 329 cases among 1,164,264 treated people — that is, 1 case of paralysis for every 3538 people treated. Owing to the incompleteness of a great many of these reports, Remlinger believed it would be more correct to assume that among this large total there were more than 500 but less than 1000 cases. Children are less frequently affected than adults. Below five years the cases are unknown. No cases have occurred during the first 5 days of treatment, and they are exceptional between the 6th and 10th day. The maximum number occur from the 13th to 15th days, after which there is a progressive diminution.

The paralyses may take the form of the Landry type of ascending paralysis, of dorsolumbar myelitis, or a purely neuritic form. The onset is usually rapid, the prognosis usually good. Most cases recover, though some observers have stated a mortality of over 20 per cent. It must be remembered, however, that in many of these cases the data given are insufficient to exclude actual death by rabies.

Yellow Fever

The brilliant researches of Walter Reed and his associates, in which the mosquito transmission of yellow fever was demonstrated, resulted in what appeared to be the complete eradication of the disease from many localities by anti-mosquito campaigns. For a long time it was believed that the disease had been exterminated from most parts of the world except for a focus in West Africa. The studies conducted by the Rockefeller Foundation during the past few years, however, indicate that this is not so. By means of a neutralization test and by histopathologic examination of liver tissue obtained by a viscerotome from individuals dying of acute illnesses, it has become evident that the disease is still

endemic in many areas. Soper (78), in particular, has described an endemic form in Brazil which had been unrecognized previously. This form of the disease is unique in that it occurs largely in isolated rural areas and is commonly referred to at the present time as "Jungle Yellow Fever."

Two discoveries in recent years have been of immense importance for a further study of yellow fever and have led directly to the development of methods of vaccination against the disease. The first of these was the discovery by Stokes, Bauer, and Hudson (79) that *Macacus rhesus* monkeys could be infected with the virus. This discovery not only provided a susceptible animal with which to work but paved the way for a conclusive demonstration that the disease is due to a filterable virus. The second discovery of paramount importance was that of Theiler (80), in which he found that white mice were susceptible to intracerebral inoculation of the virus. The repeated intracerebral passage of virus through mice resulted in the production of a neurotropic strain.

Theiler's discovery paved the way for the development of a mouse-protective test by Sawyer and Lloyd (81). This consists of the intraperitoneal inoculation of mice with a mixture of virus and immune or convalescent serum and a simultaneous intracerebral injection of a solution of starch to localize the virus. If the serum under test contains no immune bodies, the mouse dies of encephalitis. By means of this test extensive surveys have been made of the distribution of immune bodies in the sera of large numbers of people in both South America and West Africa, which sera, as we have already said, indicate widespread endemicity of yellow fever in these areas. The results of such surveys have been reported by Hughes and Sawyer (82), Soper and his associates (83), and by Beeuwkes and Mahaffy (84).

Prophylactic Immunization against Yellow Fever. The demonstration that yellow fever virus could be attenuated or changed in its properties and tissue affinities, such as the production of a neurotropic strain, naturally led to the possibility of employing such a changed virus for human vaccination. Sellards and Laigret (85) and Laigret (86) employed a neurotropic strain for human vaccination. Theiler and Whitman (87) are of the opinion that the use of such a virus alone in man is dangerous. As a result a number of methods were developed and tried, consisting of the simultaneous injection of serum and modified virus. The first attempt in this direction by the Rockefeller Foundation (88, 89) was the use of human immune serum together with a virus fixed for mice by repeated passage. Vaccination by this method was safe and produced immunity in man as well as in experimental animals.

In common with many other viruses, that causing yellow fever loses its

antigenic properties to a large extent, if not completely, when inactivated. Gordon and Hughes (90) showed that inactivation by various methods, including heating, exposure to ultraviolet light, and treatment by formaldehyde, destroyed its immunizing properties. Antigenicity was lost to such an extent that treated material was not even satisfactory for preliminary injections to be followed later by the administration of active virus. As a result, efforts have continued toward the development of an attenuated virus which could be used in an active state.

In 1932 Haagen and Theiler (91) showed that the virus of yellow fever could be grown in tissue culture, using a method similar to that developed by Rivers (92) for the cultivation of vaccinia virus. In this attempt only a virus which had been greatly modified by mouse passage was successfully cultivated. Lloyd, Theiler, and Ricci (93) later succeeded in growing an unmodified strain in tissue cultures in which mouse embryo was employed as the tissue component of the medium. They also used other tissues, such as minced chicken embryo and minced testicles of guinea pigs and mice. These studies indicated that, to a certain extent, the yellow fever virus could be attenuated by adaptation to certain tissues. Later, Theiler and Smith (94, 95) and Smith and Theiler (96) showed that by prolonged cultivation in chicken-embryo tissue culture both the viscerotropic and neurotropic properties of the virus could be attenuated. They removed the brain and spinal cord from the embryos before using them in tissue culture. As a result of these experiments, it was shown that the pathogenicity of the virus for various laboratory animals was lost to a very large extent. This changed virus was inoculated subcutaneously, without the simultaneous injection of immune serum, in four immune persons. No unusual reaction was occasioned and in two weeks following the injection a marked rise in antibody occurred. This virus was then inoculated subcutaneously in eight normal persons, who likewise experienced no unusual reaction and gradually developed immune bodies.

During the course of these studies of the cultivation of yellow fever virus, attempts were made from time to time to employ such culture material, together with various immune sera, for human vaccination. Soper and Smith (97) have reviewed these experiences. It was found that one of the most satisfactory combinations was the use of human immune serum plus tissue-culture virus. This method, however, can only be applied to small numbers because of the limited supply of suitable serum. Findlay (98) and Stefanopoulo (99) showed that immune horse serum could be employed, together with active virus, for immunization. This method had the disadvantage of producing a number of serum reactions. The Rockefeller investigators (97) employed immune goat serum plus tissue-culture virus in a series of

immunizations. This method was found unsatisfactory because of the rapid excretion of the heterologous serum. Likewise, hyperimmune monkey serum was used with tissue-culture virus. It was found that a relatively high percentage of failures followed this method, which was considered to be due to excessive amounts of antibody in the serum.

Since Theiler and Smith demonstrated the marked attenuation of the viscerotropic and neurotropic properties of the virus in their chicken-embryo tissue cultures, this material is now being used alone by the Rockefeller group for human immunization. Soper (100) has reported their most recent experiences with this method.

It has long been known that survival from an attack of yellow fever resulted in the development of a durable and permanent immunity. Naturally, of course, the study of human vaccination by the methods we have described above is as yet too young to be certain how long this immunity will last. Sawyer (101) has shown that an immune response can be demonstrated within two to three weeks after vaccination, with the peak in antibody titer occurring in about two to three months. There is then a gradual fall in titer over a period of several years. Sawyer reported observations which indicate a considerable protective titer for as long as four years, and it is not impossible that immunity following vaccination may last a great deal longer than this.

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